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**Determination and Evaluation of
Mucosal Matrix Metalloproteinase -2 and -9,
S100A12 and Myeloperoxidase in the Intestine of Dogs
with Chronic Enteropathies and Healthy Beagles**

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ACADEMIC DISSERTATION

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ABSTRACT

Chronic enteropathy (CE) in dogs refers to a group of inflammatory conditions of the intestinal tract with unknown etiology. However, the occurrence of an aberrant immune response to antigens derived from endogenous microbiota is likely to play an important role in the pathogenesis of canine CE. Thus, finding inflammatory markers that reflect disease severity would be clinically useful. Matrix metalloproteinase (MMP) -2 and -9 degrade extracellular matrix under both physiological and pathological conditions. Mucosal MMP-2 and -9 activities have been reported to be upregulated in the intestine of humans with inflammatory bowel disease (IBD) and also in animal models of human IBD. However, their identification in the intestinal mucosa of healthy Beagles and their involvement in the pathogenesis of canine CE are unknown. Elevated intestinal mucosal levels of S100A12 and myeloperoxidase, as markers of gut inflammation, have been reported in human patients with IBD. Also, increased concentrations of S100A12 in feces and serum have been reported in dogs with CE. However, intestinal mucosal S100A12 concentrations and MPO activities have not previously been investigated in dogs with CE and in healthy Beagles.

The aims of this project were to validate laboratory methods for the determination of MMP-2 and -9, S100A12, and MPO in the intestinal mucosa samples of healthy Beagles, to measure their mucosal levels in dogs with CE, and to compare these results to healthy Beagles. The project also sought to determine the relationship between the levels of the four markers and the canine clinical IBD activity index (CIBDAI), histopathologic findings, clinical outcome, and serum albumin concentrations in dogs with CE. Intestinal mucosal biopsies were collected from 40 dogs with CE (duodenum [$n = 35$], ileum [$n = 12$], colon [$n = 15$], and cecum [$n = 6$]). Stored intestinal tissue samples from 18 healthy Beagle dogs served as controls (duodenum, ileum, colon [$n = 18$, each], and cecum [$n = 6$]). MMP-2 and -9 activities, S100A12 concentrations, and MPO activities were measured using gelatin zymography, ELISA, and spectrophotometric methods, respectively. The methods for determination of MMP-2 and -9, S100A12, and MPO were successfully validated in the intestinal mucosa samples of healthy Beagles.

Compared to healthy Beagles, mucosal pro- and active MMP-2 positive samples were significantly higher in duodenum, ileum, and colon of dogs with CE, while mucosal pro-MMP-9 positive samples were significantly higher in the duodenum and colon. None of the intestinal mucosal samples in healthy Beagles showed gelatinolytic activity corresponding to the control bands of active MMP-2 and -9. In dogs with CE, however, mucosal active MMP-9 activities showed a significant positive association with the severity of neutrophils infiltration in duodenum, eosinophils in the cecum. Ileum activities were positively associated with the CIBDAI score.

Compared with healthy controls, both mucosal S100A12 concentrations and MPO activities were increased in the duodenum and colon of dogs with CE, while the mucosal MPO activity was also increased in the ileum and cecum. In dogs with CE, mucosal S100A12 concentrations had an association with the severity of epithelial injury and total histopathological injury in the colon; and with the presence of neutrophils and macrophages in the duodenal mucosa or with hypoalbuminemia. Moreover, mucosal MPO activity had a relationship with the severity of epithelial injury and total histopathological injury in the duodenum of dogs with CE.

Overall, the results of this project demonstrate an upregulation of mucosal pro- and active MMP-2 and pro-MMP-9, S100A12, and MPO in the intestine of dogs with CE compared to healthy Beagles and it seems that they are involved in the pathogenesis of canine chronic enteropathies. These results provide supporting evidence to more deeply assess the clinical utility of MMP-2 and -9, S100A12, and MPO as possible diagnostic biomarkers in dogs with CE.

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which are referred to in the text by their Roman numerals:

- I. **Hanifeh M**, Rajamäki MM, Mäkitalo M, Syrjä P, Sankari S, Kilpinen S and Spillmann T. Identification of Matrix Metalloproteinase-2 and -9 Activities within Intestinal Mucosa of Clinically Healthy Beagle Dogs. *Journal of Veterinary Medical Science*. 2014;76(8):1079-85. doi: 10.1292/jvms.13-0578.
- II. **Hanifeh M**, Heilmann R, Sankari S, Rajamäki MM, Mäkitalo L, Syrjä P, Kilpinen S, Suckodulski J, Steiner JM and Spillmann T. S100A12 concentrations and myeloperoxidase activities in the intestinal mucosa of healthy Beagle dogs. *BMC Veterinary Research*. 2015;11:234. doi:10.1186/s12917-015-0551-1.
- III. **Hanifeh M**, Rajamäki MM, Sankari S, Syrjä P, Mäkitalo M, Kilpinen S and Spillmann T. Identification of matrix metalloproteinase-2 and -9 activities within intestinal mucosa of dogs with chronic enteropathies. *Acta Veterinaria Scandinavica*. 2018;60(1):16. doi:10.1186/s13028-018-0371-y.
- IV. **Hanifeh M**, Sankari S, Rajamäki MM, Syrjä P, Kilpinen S, Suckodulski J, Steiner JM, Guadiano P, Lidbury J and Spillmann T. S100A12 concentrations and myeloperoxidase activities are increased in the intestinal mucosa of dogs with chronic enteropathies. *BMC Veterinary Research*. 2018;14(1):125. doi:10.1186/s12917-018-1441-0.

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Author's contributions

- I** The author participated in the experimental design, performed the homogenization of the mucosal samples, and collected supernatants from the homogenized samples of healthy Beagles. The author measured **protein concentrations of the supernatants**. The author also prepared polyacrylamide gels and ran the gelatin zymography method to determine MMP-2 and -9 activities. The author interpreted the results under the supervision of Thomas Spillmann and Minna Rajamaki. The author wrote and revised the manuscript.

- II** The author participated in the experimental design, performed the homogenization of the mucosal samples, and collected supernatants from the homogenized samples of healthy Beagles. The author performed spectrophotometric method for measuring MPO activities. S100A12 concentrations were measured at GI Lab, Texas A&M University, USA. The author interpreted the results under the supervision of Thomas Spillmann, Satu Sankari and Joerg Steiner. The author wrote and revised the manuscript.

- III** The author participated in the experimental design, performed the homogenization of the mucosal samples, and collected supernatants from the homogenized samples of dogs with CE and healthy Beagles. The author measured **protein concentrations of the supernatants**. The author also prepared polyacrylamide gels and ran the gelatin zymography method to determine MMP-2 and -9 activities in the mucosal samples of dogs with CE and healthy Beagles. Collecting demographic data from all dogs, and CIBDAI scores and albumin concentrations from dogs with CE were also performed by the author. The author interpreted the results under the supervision of Thomas Spillmann and Minna Rajamaki. The author wrote and revised the manuscript.

- IV** The author participated in the experimental design, performed the homogenization of the mucosal samples, and collected supernatants from the homogenized samples of dogs with CE and healthy Beagles. The author performed spectrophotometric method for measuring MPO activities. S100A12 concentrations were measured at GI Lab, Texas A&M University, USA. Collecting demographic data from all dogs, and CIBDAI scores and albumin concentrations from dogs with CE were also performed by the author. The author interpreted the results under the supervision of Thomas Spillmann, Satu Sankari and Joerg Steiner. The author wrote and revised the manuscript.

ABBREVIATIONS

ALCAM	Activated leukocyte cell adhesion molecule
ANOVA	Analysis of variance
ARD	Antibiotic-responsive diarrhea
ARE	Antibiotic-responsive enteropathy
AU	Arbitrary unit
BSA	Bovine serum albumin
CCECAI	Canine chronic enteropathy clinical activity index
CD	Crohn's disease
CE	Chronic enteropathy
CIBDAI	Canine inflammatory bowel disease activity index
cTLI	Canine trypsin-like immunoreactivity
CV	Coefficients of variation
DAMP	Damage associated molecular pattern
DSS	Dextran sodium sulfate
e.g.	exempli gratia
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-Linked immunosorbent assay
FRD	Food-responsive diarrhea
FRE	Food-responsive enteropathy
GI	Gastrointestinal
H ₂ O ₂	Hydrogen peroxide
HE	Hematoxylin and eosin
HOCl	Hypochlorous acid
HTAB	Hexadecyltrimethylammonium bromide
i.e.	id est
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
IL	Interleukin
IQR	Interquartile range
MAPK	Mitogen-activated protein kinases
MMPs	Matrix metalloproteinases
MPO	Myeloperoxidase
MT-MMPs	Membrane-type matrix metalloproteinases
NF-κB	Nuclear factor κB
O/E	Observed to expected ratios
PBS	Phosphate buffered saline
PCDAI	Pediatric Crohn's disease activity index
RAGE	Receptor for advanced glycation end products
SD	Standard deviation

SRD	Steroid-responsive diarrhea
SRE	Steroid-responsive enteropathy
SNRD	Steroid non-responsive diarrhea
SNRE	Steroid non-responsive enteropathy
TBS	Tris buffered saline
TIMPs	Tissue inhibitors of metalloproteinases
TMB	3,3',5,5'-tetramethylbenzidine substrate
TNF- α	Tumor necrosis factor-alpha
UC	Ulcerative colitis
WSAVA	World small animal veterinary association
ΔA	Delta absorbance

1 INTRODUCTION

Canine chronic enteropathy (CE) is an umbrella term for a group of inflammatory conditions of the intestinal tract with unknown etiology (Allenspach et al., 2007). Canine CE is defined by response to treatment as food-responsive diarrhea or enteropathy (FRD or FRE), antibiotic-responsive diarrhea or enteropathy (ARD or ARE), steroid-responsive diarrhea or enteropathy (SRD or SRE), or steroid-non-responsive diarrhea or enteropathy (SNRD or SNRE) (Simpson and Jergens, 2011; Dandrieux, 2016). The term inflammatory bowel disease (IBD), synonymous for CE, has also been used in dogs (Simpson and Jergens, 2011); however, since human IBD and canine CE are not similar, using the term IBD for dogs is regarded as incorrect. There is a general consensus that an unfavorable interaction between the mucosal immune system, the host genetic susceptibility and environment (e.g. microbial antigens and dietary antigens) are potential causative factors in the development of chronic gastrointestinal inflammation (German et al., 2003; Jergens et al., 2009; Simpson and Jergens, 2011; Jergens and Simpson, 2012; Cassmann et al., 2016). However, the specific pathways that lead to tissue injury and intestinal inflammation in dogs with CE are not fully understood and require further investigation (Simpson and Jergens, 2011; Schmitz et al., 2015; Cassmann et al., 2016).

Matrix metalloproteinases (MMPs) are a group of zinc- and calcium-dependent endopeptidases that proteolytically degrade extracellular matrix (ECM). They also degrade or activate a diversity of non-matrix substrates such as cytokines, growth factors, chemokines, and junctional proteins; therefore, it is likely that they play important roles in inflammatory responses (Naito and Yoshikawa, 2005; Ravi et al., 2007). Among the MMPs, MMP-2 (gelatinase A) and MMP-9 (gelatinase B) have been shown to be upregulated in the intestinal mucosa of human patients with inflammatory bowel disease (IBD) (Baugh et al., 1999; Kirkegaard et al., 2004; Gao et al., 2005; Makitalo et al., 2010) and also in animal models of human IBD (Garg et al., 2006; Ravi et al., 2007; Garg et al., 2009). MMP-2 is mainly produced by stromal cells and is believed to play a protective role against tissue damage possibly by regulation of epithelial barrier function (Garg et al., 2006; Ravi et al., 2007; Garg et al., 2009). MMP-9 is mainly produced by neutrophils and plays a crucial role in the induction of intestinal tissue inflammation through promoting neutrophils migration and defective re-epithelialization (Gao et al., 2005; Garg et al., 2006; Ravi et al., 2007; Garg et al., 2009). To our knowledge, there has been no report about MMP-2 and -9 activities in the intestinal mucosa of healthy Beagles and dogs with CE.

S100A12, also known as calgranulin C, belongs to the S100/calgranulin protein family and is mainly expressed and secreted by neutrophils (Vogl et al., 1999; Meijer et al., 2014) and macrophages/monocytes (Shiotsu et al., 2011). S100A12 appears to play a central role in innate and acquired immune responses (Foell et al., 2007).

Releasing S100A12 into the extracellular space and binding with the receptor for advanced glycation end products (RAGE) generates pro-inflammatory cytokines, induces oxidative stress, and activates nuclear factor κ B (NF- κ B). This consequently leads to amplification and perpetuation of the inflammatory response (Hofmann et al., 1999; Foell et al., 2007; Pietzsch and Hoppmann, 2009). Concentrations of S100A12 have been reported to be increased in serum, feces, and intestinal mucosal samples from human patients with IBD (Foell et al., 2003b; de Jong et al., 2006; Kaiser et al., 2007; Leach et al., 2007; Foell et al., 2008; Sidler et al., 2008; Judd et al., 2011; Dabritz et al., 2013). Increased concentrations of S100A12 have been reported in the feces and serum of dogs with CE (Heilmann et al.; Grellet et al., 2013; Heilmann et al., 2014a; Heilmann et al., 2014b; Heilmann et al., 2016b). Fecal S100A12 concentration might permit the differentiation of clinical CE subtypes, since one study showed that the parameter is higher in dogs with SRD than in dogs with FRD or ARD. SNRD dogs also carried higher S100A12 concentrations than dogs with complete remission after steroid treatment (Heilmann et al., 2016b). However, when measuring fecal S100A12 concentrations, it is impossible to differentiate the region of origin within the intestinal mucosa. Given the various physiologic roles of S100A12, it is reasonable to consider this protein's function in the intestinal mucosa during inflammation in dogs with CE. Nonetheless, there is a lack of studies determining S100A12 concentrations in the intestinal mucosa of healthy Beagles and those with CE.

Myeloperoxidase (MPO) is a peroxidase enzyme mostly found in neutrophils and at lower concentrations in monocytes/macrophages and eosinophils (Klebanoff, 2005; Roncucci et al., 2008; Preiser, 2012). MPO plays an important role in intracellular microbial destruction by producing hypochlorous acid (HOCl) from hydrogen peroxide (H_2O_2) and chloride. Extracellularly, it induces oxidative tissue damage of host tissue (Klebanoff, 2005; Odobasic et al., 2007). Mucosal MPO activity has been reported to be increased in the intestine of human patients with IBD (Kruidenier et al., 2003; Kayo et al., 2006; Hegazy and El-Bedewy, 2010; Hansberry et al., 2017) and also in animal models of IBD (Kim et al., 2012; Li et al., 2016; Lv et al., 2017). Intestinal mucosal MPO activity has not yet been investigated in healthy Beagles or dogs with CE.

It was hypothesized that zymography, ELISA and colorimetric methods can be used to reliably determine MMP-2 and -9 activities, S100A12 concentrations, and MPO activity, respectively, in canine intestinal mucosal samples. In addition, it was also hypothesized that dogs with chronic enteropathies have increased mucosal MMP-2 and -9 activities, S100A12 concentrations, and MPO activity in the intestine when compared to healthy Beagles.

Therefore, the objectives of this study were to validate laboratory methods for the determination of MMP-2 and -9, S100A12, and MPO in the intestinal mucosa samples of healthy Beagle dogs, and then measure their mucosal activities or concentrations in dogs with CE and compare the results with healthy Beagles. We also evaluated the relationship between MMP-2 and -9, S100A12, and MPO levels

with the canine IBD activity index (CIBDAI), histopathologic findings, clinical outcome, and serum albumin concentrations in dogs with CE.

2 REVIEW OF THE LITERATURE

2.1 Canine chronic enteropathies

Canine chronic enteropathy (CE) is a term used for a group of chronic diseases with unknown etiology in small and/or large intestine that may also involve the stomach (Allenspach et al., 2007; Simpson and Jergens, 2011). It causes chronic gastrointestinal symptoms such as vomiting, diarrhea, tenesmus, hematochezia, decreased appetite, and weight loss (Allenspach et al., 2007; Wennogle et al., 2017). The diagnosis of canine CE can be achieved by histologic confirmation of an idiopathic chronic inflammatory process and the response to treatment trials, such as diet change, antibiotic treatment, and anti-inflammatory drug treatment. Treatment trials in dogs with CE begin with using diet change (hydrolyzed protein diet or a protein restricted diet); and if they respond to diet change alone, they are classified as having food responsive diarrhea or enteropathy (FRD or FRE). If nonresponsive to dietary changes, antibiotic treatment commences and in case of clinical response to metronidazole/tylosin therapy, the CE is classified as antibiotic responsive diarrhea or enteropathy (ARD or ARE). Canine patients with CE that failed to respond to diet change and antibiotic treatment, but show a clinical response to glucocorticoids such as prednisolone, are classified as having steroid responsive diarrhea or enteropathy (SRD or SRE) (Allenspach et al., 2007; Simpson and Jergens, 2011). Other immunosuppressive drugs that are used to treat canine CE include azathioprine, cyclosporine, and chlorambucil (Allenspach et al., 2006; Dandrieux et al., 2013). In addition, another group of dogs with CE that fails to respond even to immunosuppressive drugs are therefore classified as steroid non-responsive diarrhea or enteropathy (SNRD or SNRE) (Simpson and Jergens, 2011; Dandrieux, 2016). FRD/FRE is the most common form of chronic enteropathies in dogs, followed by SRD/SRE and ARD/ARE (Craven et al., 2004; Allenspach et al., 2007; Allenspach et al., 2016). In contrast to dogs, human inflammatory bowel disease (IBD) has two major forms: Crohn's disease (CD) and ulcerative colitis (UC). CD is typically a disease of the ileum and the colon, forming granulomas and involving the whole intestinal wall; however, it can also affect other areas of the digestive tract. UC is an inflammatory and ulcerative disease usually limited to superficial layers (mucosa and submucosa) of the colon and histopathologically characterized by infiltration of inflammatory cells (neutrophils, lymphocytes, and plasma cells) into the rectal, colonic, and occasionally ileal mucosa (Xavier and Podolsky, 2007).

Interaction between the mucosal immune system, the host genetic susceptibility, and microbial and dietary antigens have been identified as potential causative factors in the development of chronic enteropathies in dogs (Fig. 1) (German et al., 2003;

Jergens et al., 2009; Simpson and Jergens, 2011; Jergens and Simpson, 2012; Cassmann et al., 2016). Maintaining a delicate balance between tolerance and responsiveness in the intestinal mucosal immune system is very important and disruption of this balance leads to chronic intestinal inflammation (German et al., 2003; Tanoue et al., 2010). A properly functioning mucosal immune system, the mucosal barrier, and the presence of endogenous microbiota are the main elements in the maintenance of intestinal homeostasis (Okumura and Takeda, 2016). The occurrence of an aberrant immune response to endogenous microbiota has been proposed to play an important role in the disease pathogenesis of CE in dogs (Simpson and Jergens, 2011; Schmitz et al., 2015; Cassmann et al., 2016). Thus, phagocyte activation and their biomarkers may represent potential and useful markers of inflammation in dogs with CE.

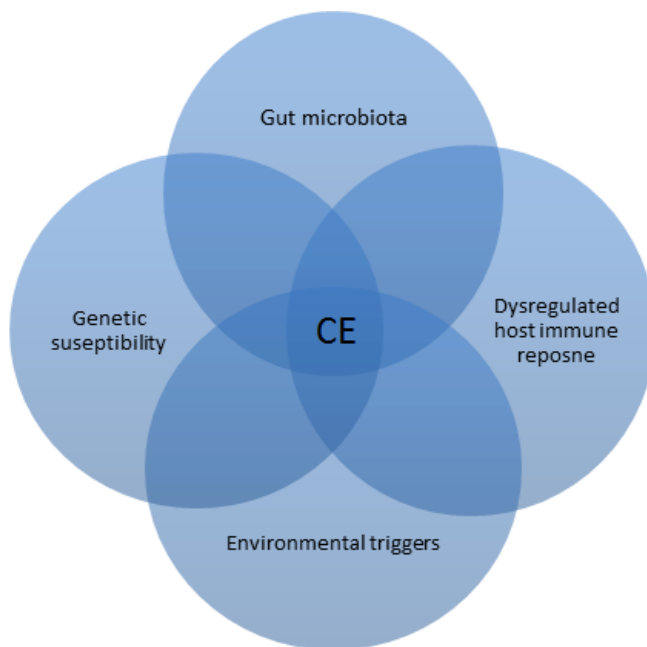


Fig. 1. Interaction between four main factors contributing to chronic intestinal inflammation. The pathogenesis of canine CE is multifactorial and dysregulated immune response by the host appears to play a central role. CE: chronic enteropathies.

2.2 Matrix metalloproteinases (MMPs)

2.2.1 Overview

Matrix metalloproteinases (MMPs) are zinc- and calcium-dependent endopeptidases which are thought to be major contributors to breakdown and reconstitution of extracellular matrix (ECM) under both physiological conditions (e.g., tissue remodeling during development and growth, intestinal epithelial-cell turnover) and pathological conditions (e.g., arthritis, atherosclerotic plaque rupture, tumor progression, and IBD) (Medina and Radomski, 2006; Makitalo et al., 2010; O'Sullivan et al., 2015).

To date, more than 24 different MMPs have been identified in humans (Pender et al., 1999; Medina and Radomski, 2006). They have been divided into subtypes based on substrate specificity and structural homology including the collagenases (MMP-1, -8, -13, and -18), gelatinases (MMP-2 and -9), stromelysins (MMP-3, -10, and -11), matrilysins (MMP-7 and -26), metalloelastases (MMP-12, -19, -20 and -28), membrane-type MMPs (MT-MMPs, including MMP-14, -15, -16, -17, -24, and -25), and others (MMP-21, -22, -23, and -27) (Table 1) (Visse and Nagase, 2003; Herouy, 2004). The MMPs are secreted as latent enzymes and become activated by the action of serine proteases and other MMPs that can cleave peptide bonds within the prodomain (Sternlicht and Werb, 2001; Medina and Radomski, 2006).

The activity of MMPs is regulated by several types of inhibitors, of which the tissue inhibitors of metalloproteinases (TIMPs) and alpha-macroglobulins are the most important (Snoek-van Beurden and Von den Hoff, 2005). TIMPs comprise a family of four protease inhibitors including TIMP 1, TIMP 2, TIMP 3, and TIMP 4 (Brew et al., 2000). TIMPs act by forming a 1:1 complex with the highly conserved zinc binding site of MMPs and the subsequent MMP-TIMP complex is inactive and unable to bind substrate (Medina and Radomski, 2006).

Table 1. Subtypes of matrix metalloproteinases (MMPs) and their substrates modified after Herouy (2004) and Kadoglou and Liapis (2004) (Herouy, 2004; Kadoglou and Liapis, 2004).

Subtypes of MMPs	MMP no.	Substrates
Collagenases		
Collagenase-1	MMP-1	Collagens (type I, II, III, VI, and X), entactin, and aggrecan
Collagenase-2	MMP-8	Collagens (type I, II, and III), aggrecan
Collagenase-3	MMP-13	Collagens (type I, II, and III)
Collagenase-4 (<i>Xenopus</i>)	MMP-18	Unknown
Gelatinases		
Gelatinase A	MMP-2	Gelatin, collagens (type I, IV, V, VII, X, and XI), fibronectin, laminin, aggrecan, elastin, tenascin C, and vitronektin
Gelatinase B	MMP-9	Gelatin, collagens (type IV, V, VII, X, and XIV), aggrecan, elastin, entactin and vitronectin
Stromelysins		
Stromelysin-1	MMP-3	Aggrecan, fibronectin, laminin, collagens (type III, IV, IX, and X), tenascin C, and vitronectin
Stromelysin-2	MMP-10	Aggrecan, fibronectin, and type IV collagen
Stromelysin-3	MMP-11	Fibronectin, laminin, type IV collagen, aggrecan
Matrilysins		
Matrilysin-1	MMP-7	Aggrecan, fibronectin, laminin, type IV collagen, elastin, entactin, tenascin, and vitronectin
Matrilysin-2	MMP-26	Type IV collagen, fibronectin, fibrinogen, and gelatin
Membrane-type MMPs		
MT1-MMP	MMP-14	Activator of prommp-2, collagens (type I, II, III), fibronectin, laminin-1, and vitronectin
MT2-MMP	MMP-15	Activator of prommp-2, fibronectin, tenascin, aggrecan
MT3-MMP	MMP-16	Activator of prommp-2, type III collagen, fibronectin
MT4-MMP	MMP-17	Unknown
MT5-MMP	MMP-24	Activator of prommp-2
MT6-MMP	MMP-25	Type IV collagen, gelatin, fibronectin, fibrin
Metalloelastase		
Macrophage Metalloelastase RASI-I	MMP-12	elastin, type IV collagen, fibronectin, laminin, vitronectin, proteoglycan
	MMP-19	aggrecan, cartilage oligomeric matrix protein
Enamelysin	MMP-20	aggrecan, cartilage oligomeric matrix protein
	MMP-28	Unknown
Epilysin		
Other MMPs		
-	MMP-21	Unknown
-	MMP-22	Unknown
-	MMP-27	Unknown

2.2.2 MMP-2 and -9 (gelatinases) and their roles in intestinal inflammation

MMP-2 and -9 are also referred to as gelatinases A and B, respectively. Both have two pro- and active forms with different molecular weights (pro-MMP-2: 72kDa, active MMP-2: 62 kDa, pro-MMP-9: 92 kDa, active MMP-9: 82 kDa). Structurally, they consist of three common domains including pro-peptide, catalytic domain, and the hemopexin-like c-terminal domain. The catalytic and hemopexin-like c-terminal domains are linked via a flexible hinge region. Unlike MMP-2, MMP-9 has an additional 56-residue domain—the type V collagen-like domain. Since MMP-2 and -9 are synthesized as pro (inactive) enzymes, cleavage of the pro-peptide (part of the cysteine switch) is essential to activate the protease. To inhibit activation of enzymes, conserved cysteine residue in the cysteine switch interacts with the zinc in the catalytic domain (Fig. 2) (Van den Steen et al., 2001; McCarty et al., 2012).

For activation of MMP-2, first, a complex of MT1-MMP/MMP-14 and TIMP-2 recruit pro-MMP-2 to the cell surface. Subsequent activation of MMP-2 requires an active MT1-MMP molecule, autocatalytic cleavage steps, cell-cell clustering, and a wild-type activated leukocyte cell adhesion molecule (ALCAM) (Lunter et al., 2005). Following the activation of pro-MMP-2, the amino terminal propeptide cleaves to generate a 64-kDa intermediate form, which later is converted to a 62-kDa active form of MMP-2 (Ravi et al., 2007).

MMP-2 degrades ECM substrates, such as gelatin, type I, IV, V, VII, X, XI collagens, elastin, laminin, and fibronectin (Table 1). It is primarily produced by stromal cells, including fibroblasts, myofibroblasts, and endothelial cells (Fig. 3) (Garg et al., 2006). Intestinal mucosal MMP-2 activities have been reported to be upregulated in humans with IBD, and also in animal models of human IBD (Baugh et al., 1999; Kirkegaard et al., 2004; Gao et al., 2005; Garg et al., 2006; Garg et al., 2009; Makitalo et al., 2010). An immunohistochemistry study in human patients with CD reported increased MMP-2 localization to epithelial cells, pericryptal and subepithelial fibroblasts and myofibroblasts, macrophages, lymphocytes, and vascular endothelial cells (Kirkegaard et al., 2004).

In an MMP-2 knockout mouse model of colitis, MMP-2 has been described to play a protective role against tissue damage possibly by regulation of epithelial barrier function (Garg et al., 2006; Ravi et al., 2007). Epithelial barrier dysfunction plays an important role in the pathogenesis of intestinal inflammation. Thus, an appropriate function of MMP-2 is a critical host factor to maintain proper epithelial barrier function and to prevent intestinal inflammation in the mouse model of IBD (Garg et al., 2006; Ravi et al., 2007). However, in humans with IBD, MMP-2 contributes to the ECM remodeling and degradation of the basal membrane type IV collagen leading to intestinal ulceration, epithelial damage, and/or fistula formation (Stallmach et al., 2000; Matsuno et al., 2003; McKaig et al., 2003; Gao et al., 2005; O'Sullivan et al., 2015).

MMP-9 is distinguished from all other MMPs by its highly glycosylated type V collagen-like domain. This extra domain affects MMP-9 substrate specificity and resistance to degradation by TIMPs. MMP-9 is also synthesized as a pro enzyme which requires proteolytic activation (Ravi et al., 2007). MMP-9 activation is achieved by an interacting plasmin and MMP-3 (stromelysin 1). Plasmin generates active MMP-3 from its zymogen and subsequently active MMP-3 cleaves the propeptide from the pro-MMP-9 (92 kDa), yielding an enzymatically active MMP-9 (Ramos-DeSimone et al., 1999). In addition to MMP-3, MMP-2 is also an important and potent activator of MMP-9. Moreover, the pro form of MMP-9 alone is able to bind and degrade certain types of gelatin (Ravi et al., 2007).

MMP-9 degrades gelatin, collagens (type IV, V, VII, X, XIV), aggrecan, elastin, entactin, and vitronectin (Table 1). It is mainly produced by neutrophils and to a lesser extent by macrophages, monocytes, eosinophils, lymphocytes, and epithelial cells (Fig. 3) (Kim et al., 2004; Gao et al., 2005; Lubbe et al., 2006; Garg et al., 2009; Hogan, 2009). Similar to MMP-2, intestinal mucosal MMP-9 activities have been reported to be upregulated in humans with IBD and also in animal models of human IBD (Baugh et al., 1999; Stallmach et al., 2000; Kirkegaard et al., 2004; Gao et al., 2005; Garg et al., 2006; Garg et al., 2009; Makitalo et al., 2010). In both humans with IBD and animal models of human IBD, MMP-9 plays a crucial role in the induction of intestinal tissue inflammation via promoting neutrophil migration, defective re-epithelialization, increased paracellular permeability, and reduction in adhesion complex integrity, resulting in impaired wound healing, especially in the acute phase (Gao et al., 2005; Garg et al., 2006; Ravi et al., 2007; Garg et al., 2009). The mechanism by which MMP-9 inhibits wound healing is unknown, however, it has been reported to play an important role in the posttranslational regulation of cadherin and occludin adhesive activities. Proteolytic cleavage of occludin or E-cadherin ectodomain by MMP-9, results in tight and adherens junction disassembly which leads to impaired cell migration and wound healing. Taken together, MMP-9 seems to play an important role in intestinal inflammation (Ravi et al., 2007).

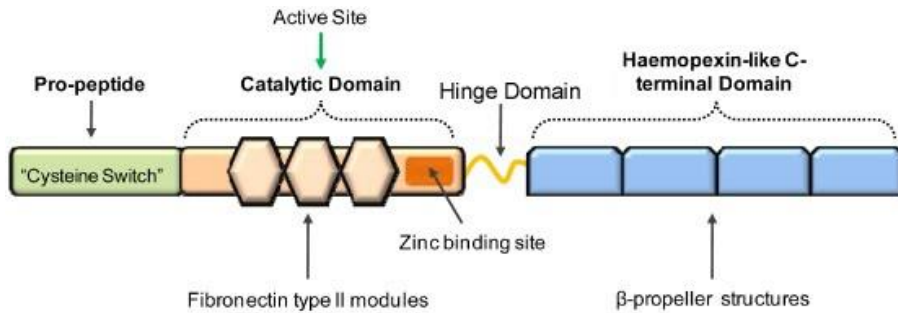


Fig. 2. Structure of MMP-2 and -9 modified after McCarty et al. (2012). Both MMP-2 and -9 have three common domains including the pro-peptide, catalytic domain, and the hemopexin-like c-terminal domain. This hemopexin region is connected to the catalytic domain through a flexible hinge domain. For activation of the pro form of MMP-2 and -9, the pro-peptide must be cleaved; and for preventing the activation of these enzymes, the cysteine switch has a conserved cysteine residue which interacts with the zinc ion in the catalytic domain.

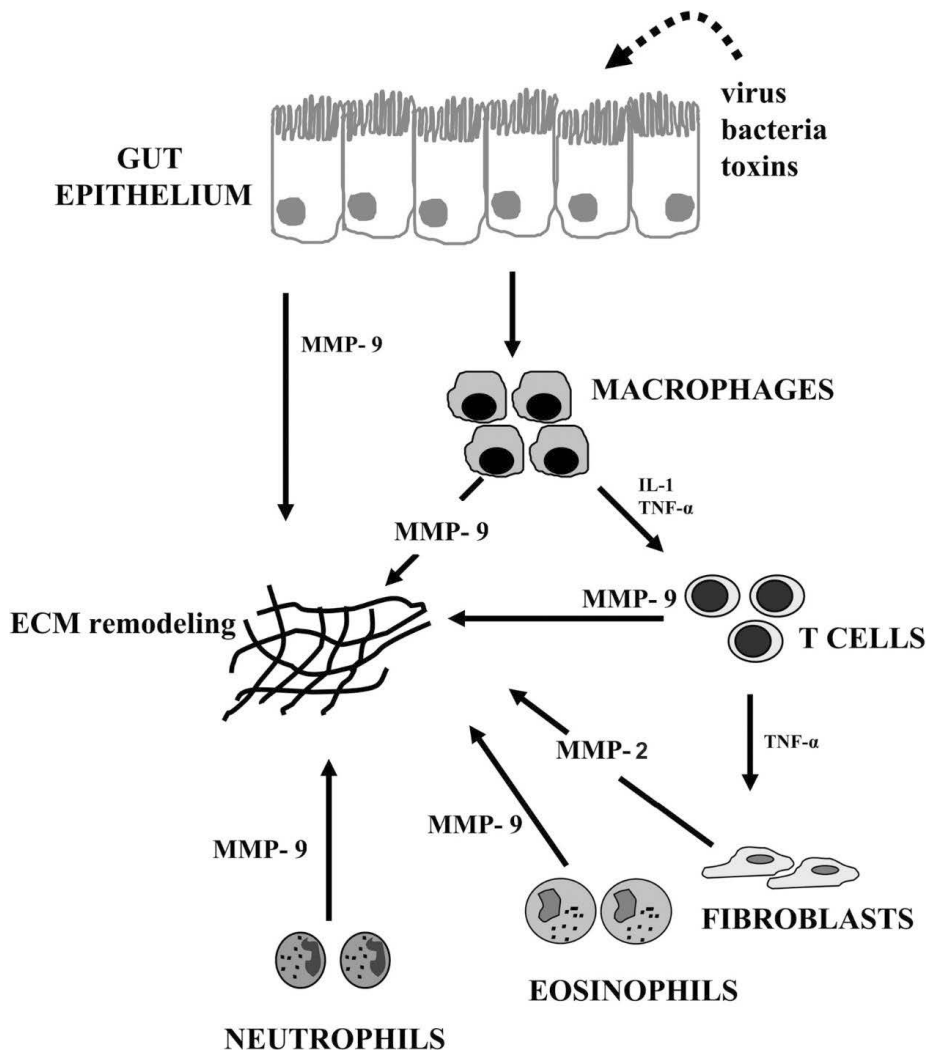


Fig. 3. MMP-2 and -9 cellular sources in human inflammatory bowel disease (IBD) modified after Medina and Radonski (2006). Virus, bacteria, or toxins in the intestinal lumen can cause immunological responses with activation of different cells, such as neutrophils, macrophages, fibroblasts, eosinophils, and T-lymphocytes. These cells release several cytokines, such as $\text{TNF-}\alpha$ and IL-1 , MMP-2 and -9, and other MMPs. In pathological conditions, such as IBD, the balance between MMPs and tissue inhibitors of metalloproteinases is disrupted, which leads to disturbed extracellular matrix remodeling. ECM: extracellular matrix; MMP: matrix metalloproteinase; $\text{TNF-}\alpha$: tissue necrosis factor- α ; Interleukin 1: IL-1 .

2.3 S100/calgranulin proteins

2.3.1 Overview

The S100/calgranulin proteins are a family of low-molecular-weight proteins consisting of at least 25 different S100 calcium-binding proteins (Manolakis et al., 2010). In addition to calcium, some S100 proteins can also bind zinc or copper (Leclerc et al., 2009). They were first identified in 1965 (Moore, 1965). The name S100 is derived from the fact that these proteins are 100% soluble in saturated ammonium sulfate at neutral pH (Meijer et al., 2012). S100 proteins are encoded by a family of genes whose symbols have the S100 as prefix (e.g., S100A1, S100A2, S100A3 etc.). All S100 proteins have two EF-hand motifs separated by a linker region (α -helix-loop- α -helix) and each motif is able to bind one calcium ion. After binding, nearly all S100 proteins undergo a conformational change leading to formation of a target recognition site which enables selective interaction with a host of specific protein or peptide targets (Leclerc et al., 2009; Meijer et al., 2012). Besides intracellular functions, three members of the S100 proteins, S100A8 (MRP8, calgranulin A), S100A9 (MRP14, calgranulin B), and S100A12 (calgranulin C) also have important extracellular activities such as anti-microbial activities, anti-fungal properties, inhibition of immunoglobulin production, neutrophil and monocyte chemotaxis, induction of apoptosis, and regulation of inflammation (Lopez et al., 2017).

2.3.2 S100A12 protein

S100A12, also known as Calgranulin C, belongs to the S100/calgranulin-protein family. S100A12 was first identified in the cytosol of human neutrophils and monocytes (Guignard et al., 1995). After first description of S100A12 protein in humans, it has also been identified later in other mammals, including dogs, pigs, cows, and rabbits (Yang et al., 1996; Yamashita et al., 1999; Chen et al., 2010; Heilmann et al., 2010). It is mainly expressed and secreted by neutrophils (Vogl et al., 1999; Meijer et al., 2014) and macrophages/monocytes (Shiotsu et al., 2011). In healthy individuals, S100A12 protein is expressed in tissues and organs where neutrophils and monocytes/macrophages are common, such as the spleen and lung (Meijer et al., 2012). S100A12 plays a key role in intracellular homeostasis and has extracellular functions such as anti-microorganism and antiparasitic activities, proinflammatory cytokine production, induction of oxidative stress, chemotaxis, and

sustained recruitment of leukocytes (Gottsch et al., 1999; Miranda et al., 2001; Pietzsch and Hoppmann, 2009). S100A12 also acts as a phagocyte-specific damage associated molecular pattern (DAMP) molecule. Thus, S100A12 appears to play an important role in innate and adaptive immune responses (Foell et al., 2007). After the release of S100A12 into the extracellular space, either due to cell damage or activation of phagocytes, it acts as a ligand for the receptor for advanced glycation end products (RAGE) (Hofmann et al., 1999; Foell et al., 2007). Binding to RAGE can induce sustained post-receptor signaling, including activation of nuclear factor κ B (NF- κ B) and the upregulation of transmembrane RAGE itself which can in turn lead to amplification and perpetuation of the inflammatory response (Hofmann et al., 1999; Schmidt et al., 2000; Foell et al., 2007; Pietzsch and Hoppmann, 2009) (Fig. 4).

S100A12 concentrations have been reported to be increased in a range of inflammatory conditions in humans including cystic fibrosis (Foell et al., 2003c), rheumatoid arthritis (Foell et al., 2003a; Foell et al., 2004), acute and chronic lung diseases (Lorenz et al., 2008), and in patients with IBD (fecal, serum, and intestinal mucosa samples) (Foell et al., 2003b; de Jong et al., 2006; Kaiser et al., 2007; Leach et al., 2007; Foell et al., 2008; Sidler et al., 2008; Judd et al., 2011; Dabritz et al., 2013). In human medicine, fecal S100A12 has been reported to be a very sensitive and specific marker for distinguishing adult IBD from irritable bowel syndrome (Yang et al.) (Kaiser et al., 2007) or pediatric active IBD from those children without IBD (Sidler et al., 2008). Serum and mucosal levels of S100A12 were increased in children with IBD as compared with non-IBD controls (Leach et al., 2007). Elevated levels of S100A12 protein in the colonic mucosa of patients with IBD imply possible contribution of S100A12 to the pathogenesis of this disease.

In dogs, canine S100A12 has been purified (Heilmann et al., 2010), and a radioimmunoassay has been developed and validated for its quantification in fecal and serum (Heilmann et al., 2011a), and urine samples (Heilmann et al., 2014c). Later, an ELISA method was also established and analytically validated for the determination of S100A12 concentrations in serum and fecal samples of healthy dogs (Heilmann et al., 2016a). S100A12 concentrations have been reported to be increased in feces and serum from dogs with CE (Heilmann et al.; Grellet et al., 2013; Heilmann et al., 2014a; Heilmann et al., 2014b; Heilmann et al., 2016b). Increased concentrations of fecal S100A12 in dogs with CE had an association with the severity of clinical signs, endoscopic lesions, colonic inflammation, and negative clinical outcome (Heilmann et al., 2014a; Heilmann et al., 2014b). Fecal S100A12 concentrations were also measured in dogs with different types of CE, including FRD, ARD, SRD, and SNRD (Heilmann et al., 2016b). Elevated levels of fecal S100A12 concentrations have been reported in dogs affected with SRD compared to those with FRD or ARD; and also in SNRD dogs compared to those experiencing complete remission after steroid therapy (Heilmann et al., 2016b). However, when measuring fecal S100A12 concentrations, it is impossible to know from which part of the intestine they originate. Given the various roles of S100A12, it is reasonable

to consider that this protein has its function in the inflamed intestinal mucosa of dogs with CE.

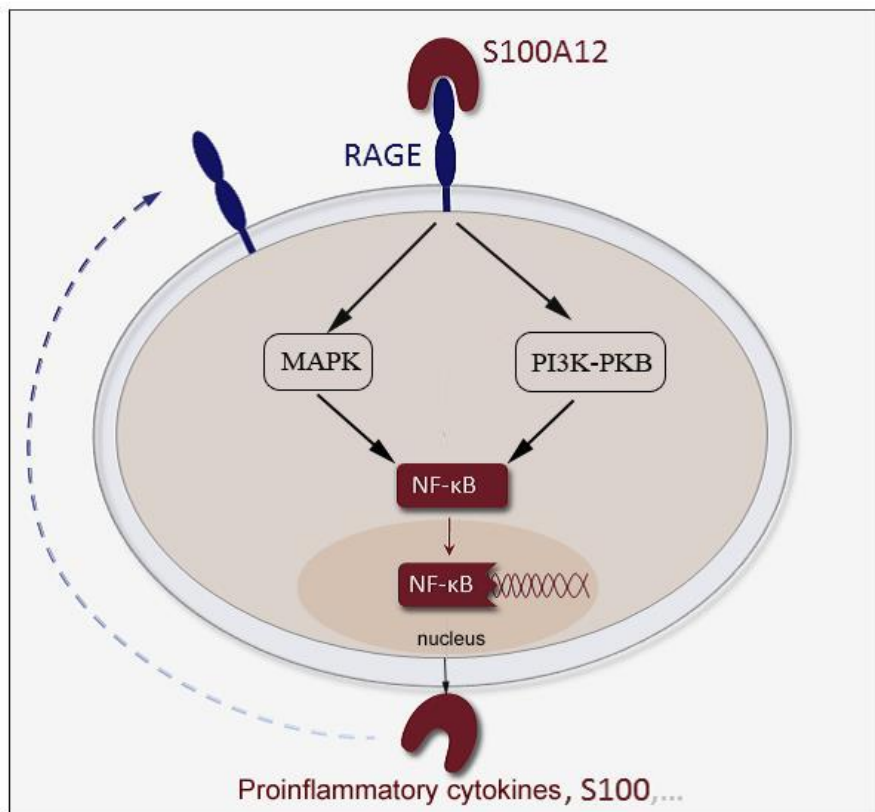


Fig. 4 . S100A12/RAGE interaction modified after Heilmann 2015, p. 21 (Heilmann, 2015). This figure shows that S100A12 by binding to the receptor of advanced glycation end products (RAGE) can induce sustained post-receptor signaling via activation and translocation of nuclear factor-kappa B (NF-κB) and the upregulation of RAGE (positive feedback loop), which, in turn, leads to amplification and perpetuation of the inflammatory response. MAPK: mitogen-activated protein kinases; PI3K-PKB: phosphatidylinositol-3-kinase-protein kinase B; NF-κB: nuclear factor-kappa B; RAGE: receptor for advanced glycation end products.

2.4 Myeloperoxidase (MPO) enzyme

Myeloperoxidase (MPO) is a peroxidase enzyme which is most abundantly expressed in neutrophils and at lower concentrations in monocytes/macrophages and eosinophils (Klebanoff, 2005; Roncucci et al., 2008; Preiser, 2012). MPO is normally stored in azurophilic granules of the neutrophil; however, it is also released into the extracellular space during degranulation. There, it generates hypochlorous acid (HOCl) from H_2O_2 and chloride (Klebanoff, 2005; Roncucci et al., 2008; Preiser, 2012). MPO also uses hydrogen peroxide to oxidize tyrosine to the tyrosyl radical. Both HOCl and tyrosyl are highly cytotoxic and can be released from the cell to destroy foreign microorganisms. However, these toxic agents can also induce oxidative tissue damage of host tissue and contribute to inflammation (Odobasic et al., 2007; Hansberry et al., 2017).

Increased MPO activity in the intestinal tissue can be utilized as a biomarker of oxidative stress and has been described in human patients with IBD (Kruidenier et al., 2003; Kayo et al., 2006; Hegazy and El-Bedewy, 2010; Hansberry et al., 2017) and also in animal models of human IBD (Kim et al., 2012; Li et al., 2016; Lv et al., 2017). Elevated levels of mucosal MPO have also been reported to be correlated with endoscopic findings in UC and with clinical activity of CD (Kayazawa et al., 2002). In addition, Saiki reported a significant elevation of MPO activity in the stool samples from active human IBD compared to inactive and healthy controls. The author also found an association between MPO with leukocyte counts and the endoscopic grade of inflammation (Saiki, 1998). Several studies have shown reduced levels of fecal MPO after treatment in human patients with IBD (Peterson et al., 2007; Wagner et al., 2008). Thus, it seems that the degree of responsiveness to IBD treatment can be monitored in people with levels of fecal MPO activity. As a result, MPO has the potential to serve as a viable, noninvasive biomarker for assessing human IBD status. These findings, however, pose the question of whether mucosal MPO activity also has a relationship with canine CE. In canine CE, the predominant inflammatory cells are thought to be lymphocytes and plasma cells. However, German et al. revealed a significant increase in the number of neutrophils and macrophages in dogs with ARD and SRD when compared to healthy controls (German et al., 2001). To our knowledge, intestinal mucosal MPO activity has not yet been investigated in dogs with CE and healthy Beagles and further research is needed to clarify its role in the pathogenesis of canine CE.

3 HYPOTHESIS AND AIMS OF THE THESIS

The main hypothesis of this thesis investigation was that MMP-2 and -9 activities, S100A12 concentrations, and MPO activities can be reliably determined using zymography, ELISA, and colorimetric methods, respectively, in canine intestinal mucosal samples. It is also hypothesized that dogs with chronic enteropathies have increased mucosal MMP-2 and -9 activities, S100A12 concentrations, and MPO activity in the intestine when compared to healthy Beagles.

The first two objectives of this PhD thesis were to validate laboratory methods for the determination of MMP-2/-9, S100A12, and MPO in the intestinal mucosa samples of healthy Beagles. The second two objectives were to measure the MMP-2/-9, S100A12, and MPO activities or concentrations in the intestinal mucosa of dogs with CE and to compare them with results of healthy Beagles. In addition, the association of MMP-2/-9, S100A12, and MPO levels with the CIBDAI, histopathologic findings, clinical outcome, and serum albumin concentrations in dogs with CE were evaluated. In this PhD thesis, it was hypothesized that mucosal MMP-2 and -9 activities, S100A12 concentrations, and MPO activities are increased in dogs with CE compared to healthy Beagles.

Detailed objectives were as follows:

- I. To validate a gelatin zymography method for the determination of MMP-2 and -9 activities in the intestinal mucosa samples of healthy Beagles.
- II. To validate ELISA and spectrophotometric methods for the determination of S100A12 concentrations and MPO activities in the intestinal mucosa samples of healthy Beagles.
- III. To investigate mucosal pro- and active MMP-2 and -9 activities in dogs with CE and healthy Beagles using gelatin zymography, and also to determine the association of their activities in CE dogs with CIBDAI, histopathologic findings, clinical outcome, and hypoalbuminemia.
- IV. To investigate mucosal S100A12 concentrations and MPO activities in dogs with CE and healthy dogs using ELISA and spectrophotometry; and to determine the association of their concentrations and activities in CE dogs with CIBDAI, histopathologic findings, clinical outcome, and hypoalbuminemia.

All four studies in this thesis are referred to by their roman numerals throughout the text.

4 MATERIALS AND METHODS

4.1 Study population and sample collection

4.1.1 Healthy Beagles (study I-IV)

For the validation of the laboratory methods, stored intestinal tissue samples were used that had been taken from 18 healthy laboratory Beagle dogs which underwent post-mortem examinations when finishing another unrelated study. The dogs were housed according to the European Union guidelines in groups in indoor pens with access to outdoor runs. The indoor environmental temperature was maintained between 15°C to 24°C. The dogs were exposed to both natural and artificial light from 7:00 to 16:00 and were fed a standard commercial diet. All dogs were considered healthy based on history, physical examination, complete blood count, serum biochemistry, fecal examination, and histologic evaluation. Immediately after euthanasia, the intestine was opened longitudinally and flushed with cold saline. For study I and II, full-thickness tissue samples were collected from duodenum, jejunum, ileum, and colon ($n = 12$, each); and for study III and IV, from duodenum, ileum, and colon ($n = 18$, each), and cecum ($n = 6$). Then, all samples were snap frozen in liquid nitrogen and stored at -80°C until further analysis. Later, the intestinal mucosa was separated from the underlying muscularis layer in the snap-frozen intestinal tissue samples and stored at -80°C for gelatin zymographic, ELISA, and spectrophotometric analyses. For histopathological examination, snap-frozen full-thickness intestinal tissue samples were first melted and fixed in formalin and then were embedded in paraffin wax.

4.1.2 Dogs with chronic enteropathies (study III and IV)

For study III and IV, 52 dogs with chronic gastrointestinal signs were enrolled into our study were enrolled into our study over a 4-year period and routine gastroduodenoscopy and/or colonoscopy were performed at the Small Animal Teaching Hospital, Faculty of Veterinary Medicine, University of Helsinki, Finland.

Inclusion criteria for canine patients were having chronic GI signs such as vomiting, diarrhea, tenesmus, hematochezia, and/or weight loss for more than 3 weeks. For each dog, diagnostic tests were performed to exclude underlying infectious or extraintestinal disorders. These tests included complete blood count, serum biochemical analysis, fecal examination for parasites, abdominal ultrasound, and gastroduodenoscopy or colonoscopy (or both) with biopsy. The diagnosis of

chronic enteropathy was based on previously published clinical, laboratory, endoscopic, and histopathologic criteria (Day et al., 2008; Washabau et al., 2010). Before starting any treatment, all dogs with chronic GI signs underwent endoscopic examination. The area of endoscopy was selected based on the clinical signs. Intestinal mucosal biopsies from dogs with chronic GI signs were collected over a 4-year period and were stored at -80°C for 1-4 years for MMP-2, MMP-9, S100A12 and MPO determinations. Group distribution and inclusion/exclusion criteria of all dogs enrolled in study III and IV are shown in Figure 5.

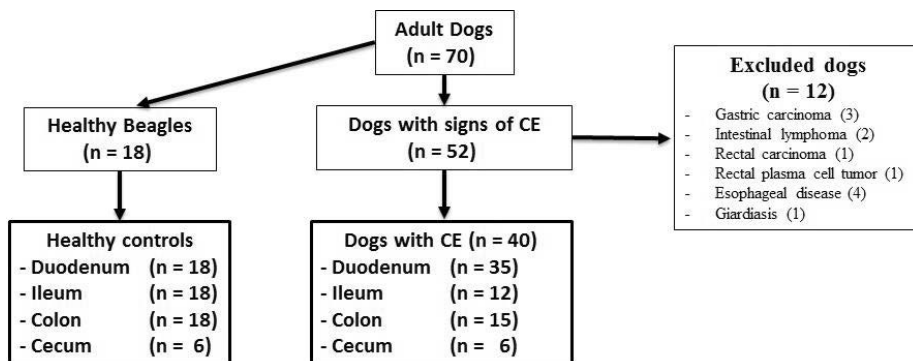


Fig. 5. Flow diagram of enrolled dogs. Flow diagram showing group distribution and inclusion and exclusion criteria of all dogs enrolled in the study. CE: chronic enteropathies

4.2 Ethical approval of study protocols

4.2.1 Healthy Beagles (study I-IV)

We used stored intestinal tissue samples taken from 12 (for study I and II) and 18 (for study III and IV) healthy laboratory Beagle dogs after finishing other non-related studies. These studies were ethically approved by the Finnish National Animal Experiment Board (study license numbers: ESLH-2007-09833/ Ym-23 ESAVI 2010-04178/Ym-23 and ESAVI/7290/04.10.03/2012).

4.2.2 Dogs with chronic enteropathies (study III and IV)

The clinical trial involving dogs with chronic enteropathies (study III and IV) were ethically approved by the same authority under the license numbers

ESAVI/6973/04.10.03/2011 and ESAVI/10384/04.10.07/2014. Informed owner consent was obtained at the time the dogs were enrolled for gastroduodenoscopy, colonoscopy, or both.

4.3 Histopathological examination (study I-IV)

For histopathological evaluation of the intestinal tissue samples from healthy Beagles, parts of the frozen intestinal tissue samples were later slowly thawed and fixed in 4% formaldehyde solution in phosphate buffered saline (PBS) at 8°C under permanent automatic rotation of the sample tube. Then, the samples were trimmed and paraffin wax embedded. Sections (3–5 µm) were prepared and stained with hematoxylin and eosin for histopathological examination. In canine patients with CE, the collected intestinal mucosal biopsy samples were fixed in 4% formaldehyde solution in phosphate buffered saline, embedded in paraffin, sectioned (3–5 µm), and stained with hematoxylin and eosin (HE) for histopathological examination.

Histopathological assessment of the intestinal samples was evaluated and scored by a single pathologist (PS) using the guide lines of the World Small Animal Veterinary Association (WSAVA) Gastrointestinal Standardization Group (Day et al., 2008; Washabau et al., 2010). In every case, the pathologist was blinded to the results of clinical and laboratory examinations and to mucosal levels of MMPs, S100A12 and MPO. In brief, for the duodenal samples, five morphological features (villous stunting, epithelial injury, crypt distention, lacteal dilation, and mucosal fibrosis) and five types of infiltrated leukocytes (intraepithelial lymphocytes, lamina propria lymphocytes, lamina propria eosinophils, lamina propria neutrophils, and lamina propria macrophages) were evaluated and scored from 0 to 3. In the colonic samples, four morphological features (epithelial injury, crypt hyperplasia, crypt dilation/distortion, and fibrosis/atrophy) and four types of infiltrated leukocytes (lamina propria lymphocytes, lamina propria eosinophils, lamina propria neutrophils, and lamina propria macrophages) were assessed and scored. The samples from ileum and cecum were examined and scored using the guidelines provided for the interpretation of duodenal and colonic biopsies, respectively; this was performed because of the absence of specific templates for these intestinal segments in the WSAVA Gastrointestinal Standardization Group guidelines (Day et al., 2008; Washabau et al., 2010). The severity of histopathological changes in different parts of the intestine was evaluated and scored as normal = 0, mild = 1, moderate = 2, or severe = 3. The total histopathological injury score was defined as the sum of the morphology and inflammatory scores and was classified as insignificant (total score 0–4), mild (total score 5–9), moderate (total score 10–14), severe (total score 15–19), or very severe (total score ≥ 20) (Day et al., 2008).

4.4 Clinical examinations of dogs with chronic enteropathies (study III and IV)

The clinical disease activity in dogs with CE was determined based on the CIBDAI scoring system at the start of the study and after treatment (Jergens et al., 2003). Briefly, CIBDAI was assessed using six prominent GI signs (i.e., attitude and activity, appetite, vomiting, stool consistency, stool frequency, and weight loss) and were scored based on their severity from 0 to 3. The total CIBDAI score represents the sum of all individual scores and was classified as insignificant (score 0-3), mild (score 4-5), moderate (score 6-8), or severe (score ≥ 9). Recording the CIBDAI score before and after treatment was only possible in 30 of 40 dogs with CE and was based on either available scores taken by the responsible clinician before and after treatment (in 13/30 and 5/30 of dogs, respectively) or calculated retrospectively by the investigators (in 17/30 and 25/30 of dogs, respectively). For retrospectively calculated scores, information was obtained from clinical history (before treatment) and phone interviews with the owners (after treatment). The treatment follow up of patients were not based on a standardized time frame and the second CIBDAI was either based on control visits or phone calls at least two weeks apart from the start of the treatment. The type of CE was determined by response to treatment and since not all included dogs developed diarrhea as a clinical sign, the CE type was defined as food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (ARE), steroid-responsive enteropathy (SRE), or steroid non-responsive enteropathy (SNRE) (Simpson and Jergens, 2011; Dandrieux, 2016). As antibiotic, Tylosin at a dose of 25mg/kg/day for 7 days was mainly used (Kilpinen et al., 2011). In some canine patients also metronidazole with/without enrofloxacin was used. For all three SNRE dogs, anallergenic diet (Royal Canin®) was started first, followed by antibiotic trial and consecutive prednisolone in two, but immediate prednisolone in the third dog. All owners opted for euthanasia due to severity of clinical signs and unfavorable response.

4.5 Serum albumin concentrations in dogs with chronic enteropathies (study III and IV)

The serum albumin concentration was determined in each dog with CE. A serum albumin concentration < 20 g/L was considered hypoalbuminemic (Allenspach et al., 2007).

4.6 Gelatin zymography for measuring MMP-2 and -9 activities (study I and III)

The analysis was performed at the Central Laboratory of the Department of Equine and Small Animal Medicine (DESAM), University of Helsinki. In the first step, snap-frozen intestinal mucosal samples from healthy Beagle dogs and dogs with CE were homogenized for 2×50 seconds with $5000 \times g$ in ice-cold extraction buffer at a ratio of 20:1 extraction buffer to tissue using Precellys 24 ceramic beads (Bertin technologies, Paris, France) (Castaneda et al., 2005). The extraction buffer contained 50 mM Tris Base, 150 mM NaCl, 10 mM CaCl₂, 0.2 mM NaN₃, and 0.01% Triton X-100 (pH 7.6) in the presence of EDTA-free protease cocktail tablets (1 tablet/50ml of extraction buffer or 1 mini tablet/10 ml extraction buffer) (Roche, Basel, Switzerland). To prevent temperature rising during the lysis process and to protect sensitive molecules from degradation, cold air (-50°C) was sprayed by Cryolys device (Bertin technologies) beside the tubes so that temperature during homogenization remained at approximately 4°C. After homogenization, samples were centrifuged at $13\,000 \times g$ at 4°C for 10 min, and the supernatants were collected and stored at -80°C for measurement of MMP-2 and MMP-9 (Medina et al., 2006). Protein concentrations of the supernatants were measured with bicinchoninic acid protein assay reagents (Pierce, Rockford, IL, USA).

Gelatinolytic activities of MMP-2 and MMP-9 in supernatant were measured by gelatin zymography in mini-gels as previously described in detail (Ljungvall et al., 2011). Supernatants were separated by electrophoresis in 11% polyacrylamide gel impregnated with 0.7 mg/ml of gelatin as a substrate (porcine skin gelatin, G-8150, Sigma, St. Louis, MO, USA) under non-reducing conditions. Each lane of an 11% SDS-polyacrylamide gel was loaded with 20 µl of supernatants containing either 10 µg or 25 µg of total protein mixed with a 10 µl aliquot of loading buffer. All samples were analyzed in duplicate and averaged. Loading buffer consisted of 0.04 g/l bromophenol blue (Art. 8122) (BDH), 20% glycerol, and 6% sodium dodecyl sulphate (SDS, Prod. 44244) (BDH) at pH 6.8. Electrophoresis was performed by using a mini-PROTEAN Tetra Cell electrophoresis system (Bio- Rad Laboratories, Hercules, CA, USA) under a constant current of 60 MA for 10 min and then 30 MA until the bromophenol blue reaches the bottom of the gel. After electrophoresis, the gels were washed in distilled water and then soaked (2×30 min) in renaturing buffer (2.5% Triton X-100) with gentle shaking at room temperature in order to remove the sodium dodecyl sulfate. Then, the gels were soaked in zymogram developing buffer (50 mM Tris Base, pH 7.5 containing 200 mM NaCl, 5 mM CaCl₂.2H₂O and 0.02% Brilj-35) for 30 min at room temperature, then replaced with fresh developing buffer and incubated for another 18 h at 37°C. Zymogram developing buffer contains

divalent metal cations, which are required for enzymatic activation of both the pro and active enzymes. After washing the gels with distilled water 3 times for 10 min, they were stained with PageBlue™ Protein Staining Solution (Fermentas) and stained with gentle agitation for 5 hr. The areas of proteinase activity were visualized as clear bands by washing the gels with distilled water.

As a control, each gel was loaded with diluted (1:600) recombinant human MMP-2 and -9 (R&D Systems, Minneapolis, MN, USA), respectively. For quantification of gelatin degradation, gels were scanned and were assessed by densitometer analysis method creating an arbitrary unit (AU) for each band by calculating the integrated area under each peak (Alpha-imager densitometer, Alpha Innotech, San Leandro, CA, USA). The activity levels of pro- and active MMP-2 and -9 for each sample were expressed in AU related to the level of pro-MMP-2 of the positive-control standard loaded on each gel. Each band's activity was reported as the mean of two different measurements of the same sample.

4.7 Enzyme-linked immunosorbent assay (ELISA) for measuring S100A12 concentrations (study II and IV)

Snap-frozen intestinal mucosal samples from dogs with CE and healthy Beagles were sent frozen to GI Lab, Texas A&M University, Texas, USA. All samples were homogenized using a Polytron homogenizer in ice-cold extraction buffer (containing 50 mM Tris/HCl base, 150 mM NaCl, 10 mM CaCl₂, 0.2 mM NaN₃ and 0.01% (v/v) Triton X-100; pH 7.6) in the presence of EDTA-free protease inhibitor cocktail tablets (1 tablet/50 ml of extraction buffer or 1 mini tablet/10 ml extraction buffer) at a ratio of 20:1 extraction buffer to tissue. After homogenization, samples were centrifuged at $13\,000 \times g$ and 4°C for 10 min, and the supernatants were collected and stored at -80°C for the measurement of S100A12.

The S100A12 concentrations were determined in the intestinal mucosal samples obtained from healthy Beagle dogs and dogs with CE using the ELISA method. The ELISA was previously developed and validated in canine serum and fecal samples (Heilmann et al., 2016a). Briefly, immunoassay plates were coated with 200 ng/well of affinity-purified polyclonal anti-canine S100A12 antibody and were blocked with 25 mM Tris-buffered saline (TBS), 150 mM NaCl, 0.05% (v/v) polyoxyethylene-20 sorbitan monolaurate, and 10% (weight/volume [w/v]) bovine serum albumin (BSA) at pH 8.0. Plates were then incubated with duplicates of standard canine S100A12 solutions, assay controls, or mucosal extracts diluted in 25 mM TBS, 150 mM NaCl, 0.05% polyoxyethylene-20 sorbitan monolaurate, and 0.5% (w/v) BSA at pH 8.0. To detect captured antigens, plates were incubated with horseradish peroxidase-labeled anti-canine S100A12 polyclonal antibody (15 ng/well), and developed with a stabilized 3,3',5,5'-tetramethylbenzidine substrate (TMB). After 5-min incubation with TMB, color development was stopped with 4 M acetic acid and 0.5 M sulfuric

acid. Absorbance was measured at 450 nm. The lower detection limit of the ELISA assay for canine S100A12 was determined by calculating the mean response + 3 times the standard deviation for 20 replicates of the blank solution. S100A12 concentrations in extracts of snap-frozen intestinal mucosal tissues were measured using the same lot of reagents for all samples and are reported as $\mu\text{g/L}$ of the intestinal mucosal supernatant.

4.8 Spectrophotometric method for measuring MPO (study II and IV)

For measuring mucosal MPO activity at the Central Laboratory of DESAM, University of Helsinki, snap-frozen intestinal mucosal samples from healthy Beagles and dogs with CE were weighed, suspended in ice-cold extraction buffer at a ratio of 20:1 of extraction buffer to tissue, and homogenized for 2×50 seconds at $5000 \times g$ using Precellys 24 ceramic beads at 4°C (Bertin technologies, France). The extraction buffer contained hexadecyltrimethylammonium bromide (HTAB; 0.5% w/v) in a 50 mM sodium phosphate buffer (pH 5.4). After homogenization, samples were centrifuged at $4000 \times g$ and 4°C for 20 min, and the supernatants were collected and stored at -80°C .

MPO activity was determined in intestinal mucosa using the method described by Marquez and Dunford (Marquez and Dunford, 1997) with the modification that the final assay buffer was supplemented with HTAB as a cationic detergent. Briefly, the reaction mixture consisted of 170 μL of sodium phosphate buffer (80 mM, pH 5.4) with HTAB (0.5% w/v) and TMB (1.6 mmol/L). Five μL of supernatant and 5 μL of distilled water were added to this reaction mixture, and the mixture was incubated at 37°C for 6 min, after which 20 μL of H_2O_2 (0.3 mmol/L) was added. After the addition of H_2O_2 , a kinetic measurement for 60 seconds was started at a wavelength of 620 nm using an automated biochemical analyzer (Konelab 30i, Thermo Fisher Scientific, Vantaa, Finland). The researchers performing the laboratory analysis (MH and SS) were blinded to the individual dog's clinical data (ie, health status, clinical outcome, and intestinal segment). The lower detection limit of the spectrophotometric assay for mucosal MPO was calculated based on the following formula: mean blank + $3 \times \text{SD}$ for 7 replicates of the blank solution. MPO activity was expressed as the delta absorbance units per minute ($\Delta\text{A}/\text{min}$) in 5 μL of supernatant.

For study II, the colorimetric method for MPO determination was validated for precision, accuracy, and recovery. Intra- and inter-assay precisions were determined as coefficients of variation calculated from six different small and large intestinal mucosa samples within one analytical run ($n = 10$) and across different runs ($n = 10$).

The linearity was evaluated using serial dilutions of the same mucosal samples (1:2, 1:4, 1:8 and 1:16) standard deviation (SD) of the observed and expected ratios (%). The spiking recovery was analyzed among six different spiking activity samples of human MPO-pure enzyme (0.066, 0.109, 0.161, 0.191, 0.318 and 0.477 $\Delta A/\text{min}$) in five different small and large intestinal mucosa samples. The mean and SD values were calculated for the observed and expected ratios (%).

4.9 Statistical analysis

For all analyses, we considered values of $P < 0.05$ as significant. All statistical analyses were performed using the SAS 9.3 statistical software (SAS Institute Inc., Cary, NC, USA).

4.9.1 Study I

The differences between the separate parts of the intestine (duodenum, jejunum, ileum and colon) in their proportions of samples with pro-MMP-2 and -9 activities were analyzed with exact McNemar's test. Bonferroni adjustment was used to control for multiplicity issues. Data are presented as number (%) or median (range) as appropriate.

4.9.2 Study II

Data are presented as medians (interquartile range). The differences in the S100A12 concentrations and MPO activities between the four different segments of the intestine (duodenum, jejunum, ileum, and colon) were analyzed using an analysis of variance (ANOVA) model. To satisfy the assumptions of normality and the homogeneity of variances, the original values were transformed to a logarithmic scale. If the results of the Levene's test were significant ($P < 0.05$) following data transformation, an ANOVA-Welch test was used for further analysis. The fitted model included the section of intestine as the fixed effect and the dog as the random effect. Tukey's HSD test was used in pairwise comparisons to control for multiple comparisons, and Tamhane's T2 test was used for variables with significant differences in the variance.

4.9.3 Study III

Data are presented as number (%) or median (range) as appropriate. The differences between pro- and active MMP-2 and -9 positive samples in different intestinal segments (duodenum, ileum, colon, and cecum) between dogs with CE and healthy Beagles were analyzed using Fisher's exact test. Association of CIBDAI score and pro- and active MMP-2 and -9 activities was evaluated using Spearman correlation test. The same test was used to analyze correlation between hypoalbuminemia and pro- and active MMP-2 and -9 activities in dogs with CE. Kruskal-Wallis test was used to analyze association between pro- and active MMP-2 and -9 activities and histopathological changes and clinical outcome (FRE, ARE, SRE, and SNRE) in dogs with CE.

4.9.4 Study IV

To satisfy the assumptions of normality and the homogeneity of variances, the original values of S100A12 and MPO were transformed to a logarithmic scale. The normality of log-transformed values was then confirmed by Shapiro-Wilk test. The differences between S100A12 concentrations and MPO activities in different intestinal segments (duodenum, ileum, colon, and cecum) between dogs with CE and healthy Beagles were determined using Student's *t*-tests. An analysis of variance (ANOVA) test was used to determine the association between S100A12 concentrations and MPO activities with total histopathological injury severity (insignificant, mild, moderate, or severe) and individual histopathological changes severity (normal, mild, moderate, or severe). A Spearman's correlation coefficient was used to determine a potential correlation between S100A12 concentrations and MPO activities in dogs with CE and healthy Beagles. A possible correlation between CIBDAI score and S100A12 concentrations or MPO activities was tested using linear regression. The generalized logit-model was used to determine a possible association between S100A12 concentrations and MPO activities with clinical outcome (ie, FRE, ARE, SRE, or SNRE) in dogs with CE. All statistical differences were calculated based on the log-transformed values of S100A12 and MPO, and presented as mean and standard deviation in figures. However, untransformed values are presented as median (interquartile range: IQR) or median (range) as appropriate, for ease of interpretation in tables and texts. No adjustment was made for multiple comparisons in this study.

5 RESULTS

5.1 Study population

5.1.1 Study I and II

The median age (range) of healthy Beagle dogs was 11 years (10-13), 8 dogs were intact females and 4 dogs were intact males.

5.1.2 Study III and IV

The median age (range) of dogs with CE and healthy Beagles were 5 years (1–13 years) and 10.5 (6-13), respectively. In the CE group, 15 dogs were intact males, 11 dogs were castrated males, 5 dogs were intact females, and 9 dogs were spayed females. In the healthy Beagle control group, 8 dogs were intact males and 10 dogs were intact females. The breeds of dogs with CE were mixed breed (6), Rottweiler (2), German Shepherd dog (2), Shetland Sheepdog (2), Parson Russell Terrier (2), Rough Collie (2), Standard Poodle (2), and one each of the following: Alaskan Malamute, Bichon Frise, Border Terrier, Chow Chow, Dalmatian, English Bulldog, Golden Retriever, Havanese, Irish Terrier, Jack Russell Terrier, Long-haired Dachshund, Mudi, Norwegian Lundehund, Rhodesian Ridgeback, Siberian Husky, Silky Terrier, Smooth Collie, Spanish Water Dog, Staffordshire Bull Terrier, Toy Poodle, West Highland White Terrier, and White Shepherd dog.

Out of 52 dogs, 12 displaying chronic GI signs were excluded from further analysis. Seven dogs had gastrointestinal neoplasia (ie, 3 gastric adenocarcinomas, 2 lymphomas, 1 rectal plasma cell tumor, and 1 rectal adenocarcinoma). Four dogs had primary esophageal disorders, and one was positive for *Giardia* on fecal examination. A total of 40 dogs with CE were included in the study for analysis. Twenty-five dogs underwent gastroduodenoscopy, 10 dogs had both gastroduodenoscopy and colonoscopy, and colonoscopy alone was performed in 5 dogs. Sixty-eight intestinal mucosal biopsies were collected by endoscopy from four different segments of the intestinal tract of above-mentioned dogs, namely duodenum ($n = 35$), ileum ($n = 12$), colon ($n = 15$), and cecum ($n = 6$).

5.2 Mucosal MMP-2 and -9 activities in healthy Beagle dogs (study I)

Gelatin zymography was performed with 48 mucosal samples from four different intestinal segments (duodenum, jejunum, ileum, and colon) of 12 healthy Beagles. When positive, it showed gelatinolytic activity at the same level as the positive control bands of pro-MMP-2 and -9 and was therefore considered to represent canine pro-MMP-2 and pro-MMP-9 (Fig. 6). Since the majority of samples containing 10 µg of total protein showed no activity (pro-MMP-2: 40/48, 83%; pro-MMP-9: 33/48, 69%), only the results for samples containing 25 µg of total protein are presented here. Pro-MMP-2 and -9 activities were found in 17/48 (35%) and 25/48 (52%) of the samples, respectively. None of the intestinal samples showed gelatinolytic activity corresponding to the control bands of active MMP-2 and MMP-9.

Using McNemar test, no significant difference was found for pro-MMP-2 and -9 activities between the four separate parts of the intestine (duodenum, jejunum, ileum and colon) ($P > 0.05$). However, for pro-MMP-2, the number (and percentage) of positive samples in four different parts of the intestine of 12 dogs were from the highest to the lowest as follows: ileum, 7/12 (58.3%); jejunum, 5/12 (41.7%); duodenum, 3/12 (25%); and colon, 2/12 (16.7%). For pro-MMP-9, ileum had the highest positivity rate (8/12; 66.7%), followed by jejunum (6/12; 50%), duodenum (6/12; 50%), and colon (5/12; 41.7%). From all 48 canine intestinal mucosa samples examined with zymography, no pro-MMP-2 and -9 activity was found in 31/48 (65%) and 23/48 (48%), respectively.

The enzyme activities ranged for pro-MMP-2 between 0.015 and 6.449 AU and for pro-MMP-9 between 0.018 and 5.680 AU. In the examined four different parts of the intestine, the median (range) pro-MMP-2 activity was from the highest to the lowest: ileum, 0.175 (0-3.337) AU; jejunum, 0 (0-6.449) AU; duodenum, 0 (0-4.301) AU; and colon, 0 (0-0.146) AU. The highest pro-MMP-9 activity [median and range] was found in the ileum (0.086 [0-1.443] AU), followed by colon (0.018 [0-1.652] AU), jejunum (0.011 [0-2.810] AU) and duodenum (0 [0-5.680] AU). In the majority of positive samples (36/42; 86%), either pro-MMP-2 or -9 activities were lower than 2 AU throughout the whole intestine (Table 2). Comparably high activities ($AU \geq 2$) of pro-MMP-2 were recorded in two mucosa samples from duodenum and jejunum, as well as one ileum. Two duodenal mucosa samples and one jejunum sample presented the highest activities of pro-MMP-9 (Table 2).

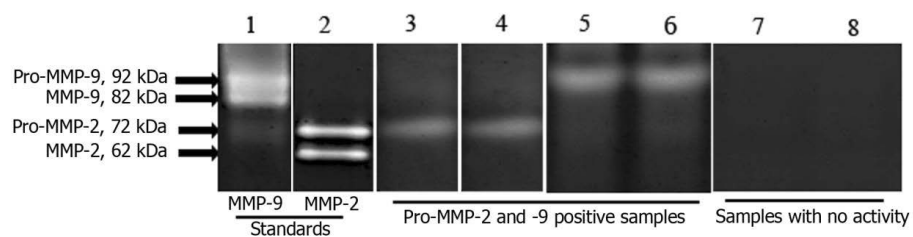


Fig.6. Representative zymogram from intestinal mucosa samples of healthy Beagles (25 μ g protein). Recombinant human pro- and active MMP-2 (lane 2) and -9 (lane 1), pro-MMP-2 and -9 positive samples (lanes 3–6) and samples with no activity (lanes 7 and 8). (Study I)

Table 2. Distribution of pro-MMP-2 and -9 gelatinolytic activities in mucosa samples from duodenum, jejunum, ileum, and colon of 12 healthy Beagles;
AU: arbitrary units, Pro: pro-enzyme

Intestinal part	Dog's No Variable	1	2	3	4	5	6	7	8	9	10	11	12
Duodenum	Pro-MMP-2 (AU)	0	0	0	0	4.30	0	0	0	0.51	1.46	0	0
	Pro-MMP-9 (AU)	0.23	0	0	0	0	0.04	0	0	5.68	5.42	0.62	0
Jejunum	Pro- MMP-2	0	0	0	0	6.45	0.58	1.53	0.23	0.23	0	0	0
	Pro-MMP-9	0.02	0	0	0	0	0	1.09	0.18	2.81	0.02	0	0.73
Ileum	Pro- MMP-2	0.15	0.29	0	0	0	0.56	0	3.34	0.20	0.50	0	1.88
	Pro-MMP-9	0.59	0	1.44	0.06	0	0.10	0	0.62	0.18	0.08	0.09	0
Colon	Pro- MMP-2	0.15	0	0	0	0	0	0	0	0	0	0	0.03
	Pro-MMP-9	1.65	0	0.14	0.09	0	0	0	0	0.04	0.05	0	0.04

5.3 Mucosal measuring MMP-2 and -9 activities in dogs with CE (study III)

Zymographic analyses revealed that gelatinolytic activities in positive samples were at the same molecular weights as the positive control bands of pro- and active MMP-2 and -9, and were therefore considered to represent canine pro- and active MMP-2 and -9 (Fig. 7). The median and range of mucosal pro- and active MMP-2 and -9 activities in each intestinal segment in dogs with CE and healthy Beagles are shown in Table 3.

In the duodenum, dogs with CE compared to healthy Beagles had a significantly higher number [and percentage] of samples positive for mucosal pro-MMP-2 (32/35 [91.4%] vs. 3/18 [16.7%]; $P < 0.0001$), active MMP-2 (10/35 [28.6%] vs. 0/18 [0%]; $P = 0.0108$), and pro MMP-9 (34/35 [97.1%] vs. 10/18 [55.6%]; $P = 0.0004$) (Fig. 8). For active MMP-9, two positive samples were recorded in dogs with CE compared to none in healthy Beagles, but this difference was not significant (2/35 [5.7%] vs. 0/18 [0%]; $P = 0.5428$) (Fig. 8). None of the intestinal mucosal samples in healthy Beagles showed gelatinolytic activity corresponding to the control bands of active MMP-2 and -9.

In the ileum, dogs with CE compared to healthy Beagles had significantly higher numbers [and percentage] of samples positive for mucosal pro-MMP-2 (11/12 [91.7%] vs. 6/18 [33.3%]; $P = 0.0024$) and active MMP-2 (4/12 [33.3%] vs. 0/18 [0%]; $P = 0.0181$; Fig. 9). For pro- and active MMP-9, dogs with CE had more positive samples compared to healthy Beagles, but the differences were not significant (11/12 [91.7%] vs. 11/18 [61.1%], $P = 0.0994$ and 3/12 [25%] vs. 0/18 [0%]; $P = 0.0542$, respectively) (Fig. 9).

In the colon, dogs with CE compared to healthy Beagles had significantly higher number [and percentage] of samples positive for mucosal pro-MMP-2 (13/15 [86.7%] vs. 3/18 [16.7%]; $P < 0.0001$), active MMP-2 (7/15 [46.7%] vs. 0/18 [0%]; $P = 0.0015$), and pro-MMP-9 (15/15 [100%] vs. 8/18 [44.4%]; $P = 0.0005$, Fig. 10). In dogs with CE only, however, one had active MMP-9 positive samples compared to healthy Beagles with none and the difference was not significant (1/15 [6.7%] vs. 0/18 [0%]; $P = 0.4545$) (Fig. 10).

In the cecum, despite a higher percentage of samples positive for pro- and active MMP-2 and -9 in dogs with CE compared to healthy Beagles, the differences between them did not reach statistical significance (Fig. 11).

Table 3. Mucosal pro- and active MMP-2 and -9 activities in dogs with CE and healthy Beagles. Pro- and active MMP-2 and -9 activities were measured in the mucosal samples from duodenum, ileum, colon, and cecum of dogs with CE and healthy Beagles. AU: arbitrary units; CE: chronic enteropathies; Pro: pro-enzyme

Intestinal part	Activity Group	Pro-MMP-2 (AU) Median (range)	Active MMP-2 (AU) Median (range)	Pro-MMP-9 (AU) Median (range)	Active MMP-9 (AU) Median (range)
Duodenum	Dogs with CE (n = 35)	0.01 (0–1.66)	0 (0–0.02)	0.06 (0–3.86)	0 (0–0.21)
	Healthy Beagles (n = 18)	0 (0–0.64)	0 (0–0)	0.03 (0–3.32)	0 (0–0)
Ileum	Dogs with CE (n = 12)	0.02 (0–0.83)	0 (0–0.01)	0.06 (0–0.5)	0 (0–0.28)
	Healthy Beagles (n = 18)	0 (0–0.52)	0 (0–0)	0.03 (0–0.45)	0 (0–0)
Colon	Dogs with CE (n = 15)	0.03 (0–0.55)	0 (0–0.05)	0.07 (0.02–0.92)	0 (0–0.04)
	Healthy Beagles (n = 18)	0 (0–0.64)	0 (0–0)	0 (0–0.79)	0 (0–0)
Cecum	Dogs with CE (n = 6)	0.0 (0.01–1.48)	0 (0–0.01)	0.09 (0.02–0.59)	0.005 (0–0.05)
	Healthy Beagles (n = 6)	0 (0–0.3)	0 (0–0)	0.18 (0–0.82)	0 (0–0)

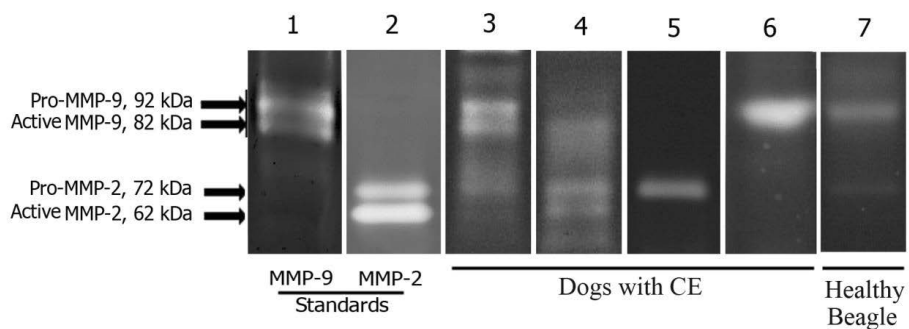


Fig. 7. Representative zymogram from intestinal mucosa samples (10 µg protein). Recombinant human pro- and active MMP-2 (lane 2) and -9 (lane 1), pro- and active MMP-2 and -9 positive samples in dogs with CE (lanes 3–4), pro-MMP-2 and -9 positive samples in dogs with CE (lanes 5–6), and a healthy Beagle (lane 7). (Study III)

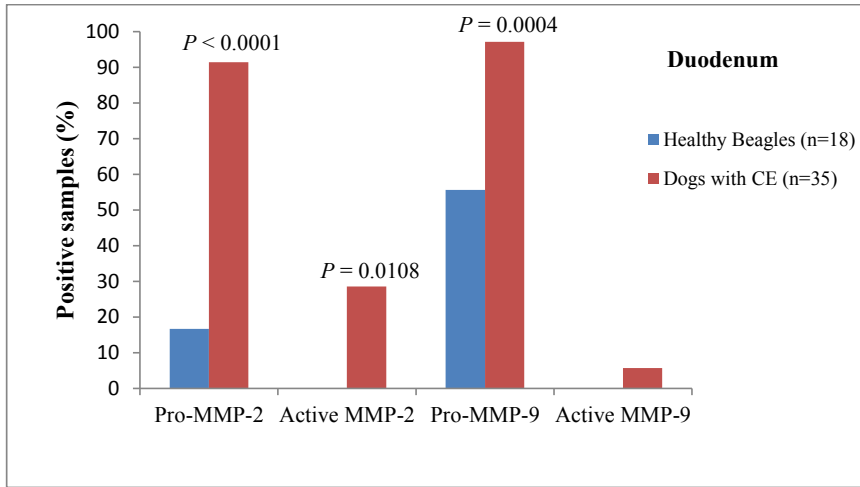


Fig. 8. The presence of samples positive for pro- and active MMP-2 and -9 in the duodenal mucosa of dogs with CE and healthy Beagles. *P*-values are based on the comparison of pro- and active MMP-2 and -9 activities in the duodenum of dogs with CE versus healthy Beagles. (Study III)

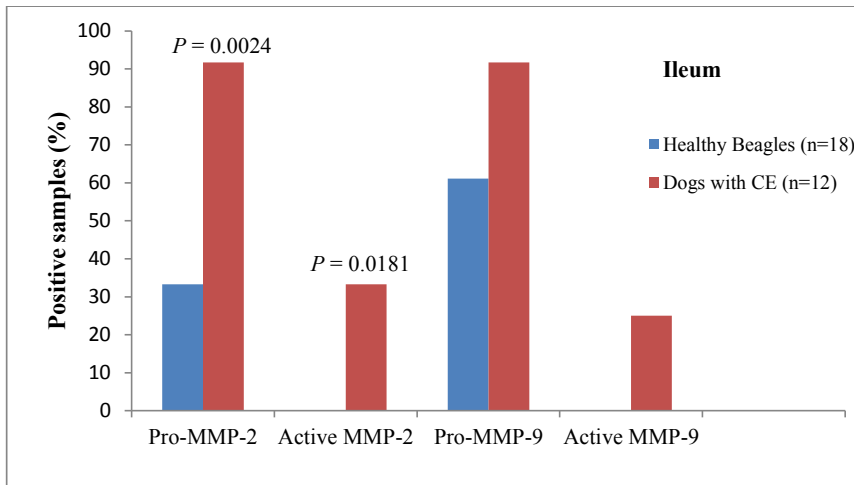


Fig. 9. The presence of samples positive for pro- and active MMP-2 and -9 in the ileal mucosa of dogs with CE and healthy Beagles. *P*-values are based on the comparison of pro- and active MMP-2 and -9 activities in the ileum of dogs with CE versus healthy Beagles. (Study III)

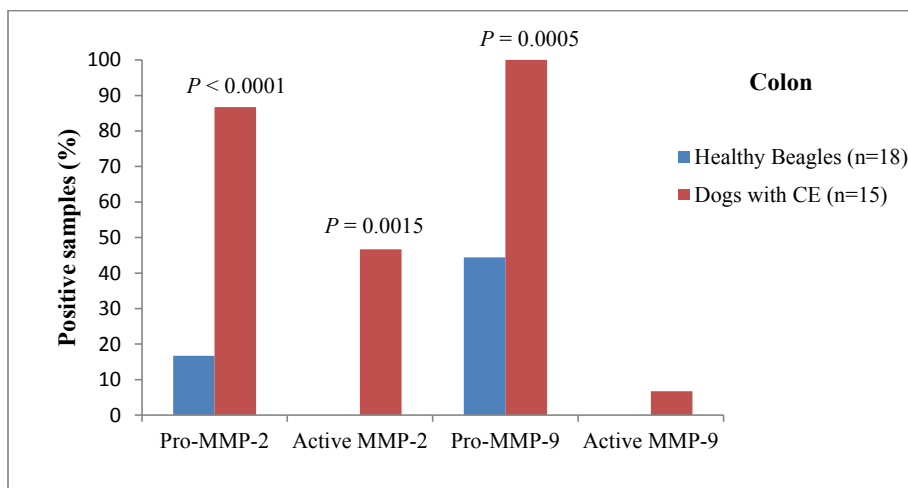


Fig. 10. The presence of samples positive for pro- and active MMP-2 and -9 in the colonic mucosa of dogs with CE and healthy Beagles. *P*-values are based on the comparison of pro- and active MMP-2 and -9 activities in the colon of dogs with CE versus healthy Beagles. (Study III)

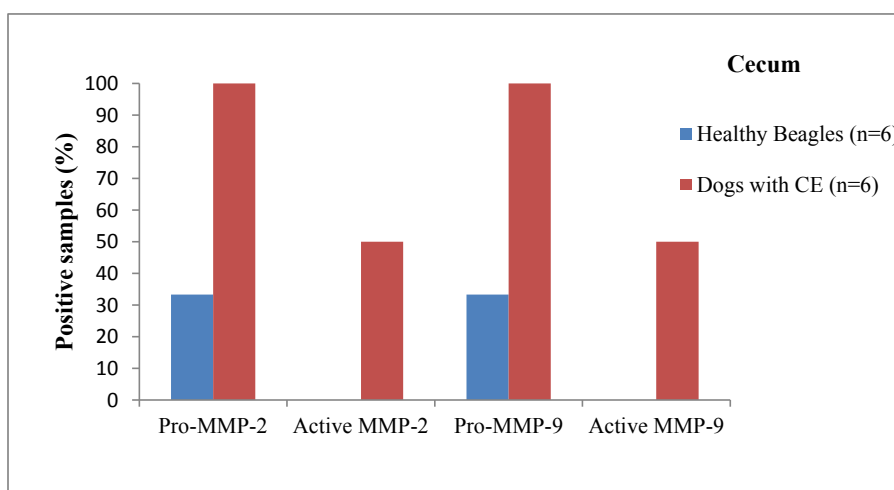


Fig. 11. The presence of samples positive for pro- and active MMP-2 and -9 in the cecal mucosa of dogs with CE and healthy Beagles. Despite a higher percentage of samples positive for pro- and active MMP-2 and -9 in cecal samples in dogs with CE compared to healthy Beagles, the differences between them did not reach statistical significance. (Study III)

5.4 Mucosal S100A12 concentrations in healthy Beagle dogs (study II)

The S100A12 concentration in all samples ranged from 2.5 to 237.6 µg/L. In the four different parts of the intestine examined in 12 healthy Beagles, the highest median levels of S100A12, which were significant, were found in the ileum (71.5 µg/L [38.9–141.9]), followed by the colon (23.2 µg/L [6.7–75.6]), duodenum (11.4 µg/L [6.9–28.5]), and jejunum (8.5 µg/L [5.1–19.3]) (Table 4 and Fig. 12). In addition, the difference between the colonic and jejunal mucosa was significant ($P < 0.05$).

Table 4. Distribution of S100A12 concentrations and MPO activities in the intestinal mucosal samples of 12 healthy Beagles.

Intestinal segment	Dog's No	1	2	3	4	5	6	7	8	9	10	11	12
	Variable												
Duodenum	S100A12 (µg/L)	6.0	27.9	7.3	5.4	27.0	45.8	28.7	14.0	30.3	8.7	6.7	8.0
	MPO (ΔA/min)	0.61	0.35	0.46	0.03	0.17	0.09	0.70	0.08	0.11	0.62	0.54	0.10
Jejunum	S100A12 (µg/L)	7.3	22.1	2.5	5.0	31.3	20.8	9.6	5.5	11.9	5.8	2.7	14.8
	MPO (ΔA/min)	5.12	1.04	2.15	0.22	0.26	0.32	1.92	0.37	0.22	0.35	0.85	0.36
Ileum	S100A12 (µg/L)	38.1	143.0	41.2	18.3	91.6	209.6	138.4	90.8	237.6	52.1	44.1	20.0
	MPO (ΔA/min)	0.18	1.14	1.25	0.16	0.80	1.33	1.43	0.18	0.22	0.72	0.55	0.28
Colon	S100A12 (µg/L)	11.5	123.4	64.4	5.2	79.3	57.6	4.2	6.8	34.9	94.4	6.6	7.0
	MPO (ΔA/min)	0.20	0.12	0.14	0.06	0.10	0.14	0.03	0.05	0.04	0.52	0.03	0.09

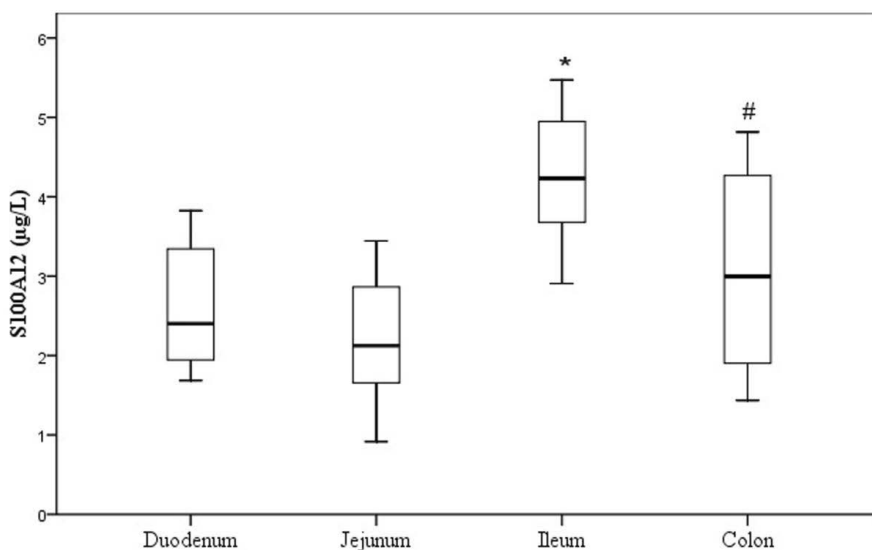


Fig. 12. Boxplot representing the log-transformed S100A12 concentrations in the intestinal mucosa samples of 12 healthy Beagles. The horizontal line inside each box represents the median; the top and bottom of each box represent the 75th and 25th percentiles, respectively; and the whiskers represent the 95th and 5th percentiles. * $P < 0.05$ vs. duodenum, jejunum, and colon; # $P < 0.05$ vs. jejunum. (Study II)

5.5 Mucosal S100A12 concentrations in dogs with CE (study IV)

Mucosal S100A12 concentrations in each intestinal segment in dogs with CE and healthy Beagles are shown in Figure 13. The median (IQR) of mucosal S100A12 concentrations in dogs with CE were significantly higher than those in healthy Beagles in the duodenum (43.93 [23.62–78.03] vs. 11.86 [7.66–29.1] µg/L; $p < 0.0001$) and colon (63.04 [33.53–211.53] vs. 15.94 [6.95–59.3] µg/L; $P = 0.0011$). Even though dogs with CE had higher mucosal S100A12 concentrations than healthy Beagles in the ileum (118.54 [47.26–142.9] vs. 48.1 [24.87–91.4] µg/L; $P = 0.2725$) and cecum (160.38 [43.27–326.28] vs. 33.53 [27.97–38.01] µg/L; $P = 0.2194$), these differences did not reach statistical significance.

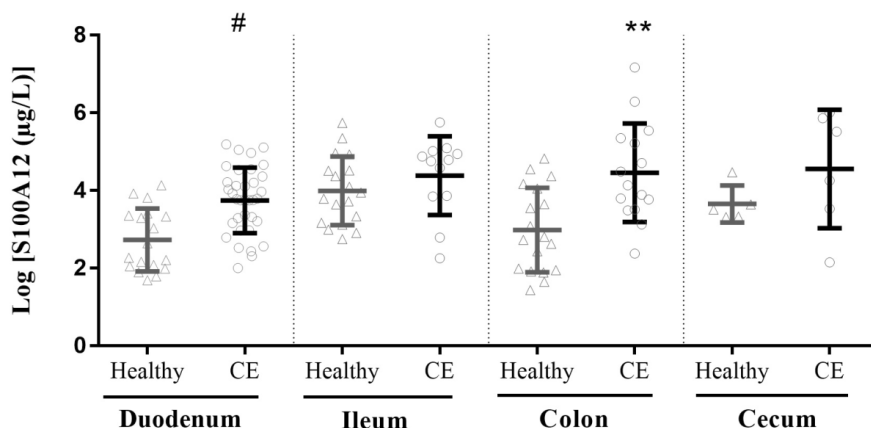


Fig. 13. Scatter plot displaying log-transformed intestinal mucosal S100A12 concentrations in CE dogs and healthy Beagles. Data are expressed as the mean \pm standard deviation. Individual data points are shown by triangles (samples from healthy Beagles) and circles (samples from CE dogs). CE: chronic enteropathies; $**P < 0.01$ vs. healthy colon; $\#P < 0.0001$ vs. healthy duodenum. (Study IV)

5.6 Analytical validation of spectrophotometric method for MPO measurement (study II)

The intra-assay coefficients of variation (CV%) in six different small intestinal mucosa samples assayed in a 1:2 dilution were 0.77, 0.85, 1.80, 1.56, 0.64, and 1.02%, and were 1.55, 1.84, 1.24, 1.30, 1.03, and 1.03% in six different large intestinal mucosa samples tested in a 1:2 dilution. The inter-assay CV% for six different small intestinal and six different large intestinal mucosa samples assayed in a 1:2 dilution were 1.77, 0.37, 2.55, 2.24, 1.32, and 2.59%, and 7.00, 7.59, 1.76, 2.74, 1.00, and 1.66%.

The observed to expected ratios (O/E) at dilutions of 1:2, 1:4, 1:8, and 1:16 ranged from 91.6–106.9% (mean \pm SD: $98.3 \pm 3.8\%$) for six different small intestinal mucosa samples, and from 84–103.8% ($93.6 \pm 4.9\%$) for six different large intestinal mucosa samples. O/E for the spiking recovery ranged from 97.3–111.1% ($103.7 \pm 4.8\%$) for five different small intestinal mucosa samples, and from 90.2–103.8% ($97.1 \pm 4.4\%$) for five different large intestinal mucosa samples.

5.7 Mucosal MPO activity in healthy Beagle dogs (study II)

The MPO enzyme activity was determined in all mucosal samples from four different intestinal areas of 12 healthy Beagles. The median MPO activity was significantly higher in the ileum (0.49 $\Delta A/\text{min}$ [0.19–1.05]), jejunum (0.36 $\Delta A/\text{min}$ [0.28–1.70]) and duodenum (0.26 $\Delta A/\text{min}$ [0.09–0.59]) than in the colon (0.09 $\Delta A/\text{min}$ [0.04–0.14]) ($P < 0.05$) (Table 4 and Fig. 14). In addition, the difference between the jejunal and duodenal mucosa was significant ($P < 0.05$) (Fig. 14).

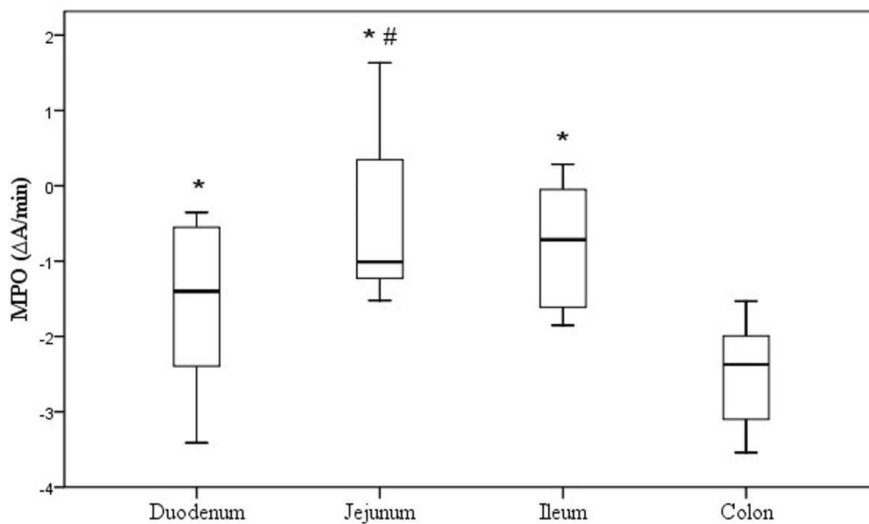


Fig. 14. Boxplot representing the log-transformed MPO activities in the intestinal mucosal samples of 12 healthy Beagles. The horizontal line inside each box represents the median; the top and bottom of each box represent the 75th and 25th percentiles, respectively; and the whiskers represent the 95th and 5th percentiles. * $P < 0.05$ vs. colon; # $P < 0.05$ vs. duodenum. (Study II)

5.8 Mucosal MPO activity in dogs with CE (study IV)

Mucosal MPO enzyme activity in each intestinal segment in dogs with CE and healthy Beagles are shown in Figure 15. The median (IQR) of mucosal MPO activity of dogs with CE was significantly higher than the corresponding activity in healthy Beagles in the duodenum (1.3 [0.77–2.16] vs. 0.41 [0.11–0.64] $\Delta A/\text{min}$; $P < 0.0001$), ileum (1.91 [0.72–2.83] vs. 0.75 [0.21–1.27] $\Delta A/\text{min}$; $P = 0.0083$), colon

(1.46 [0.57–3.01] vs. 0.09 [0.03–0.17] $\Delta A/\text{min}$; $P < 0.0001$) $\Delta A/\text{min}$), and cecum (0.68 [0.3–1.55] vs. 0.19 [0.08–0.4] $\Delta A/\text{min}$; $P = 0.0474$).

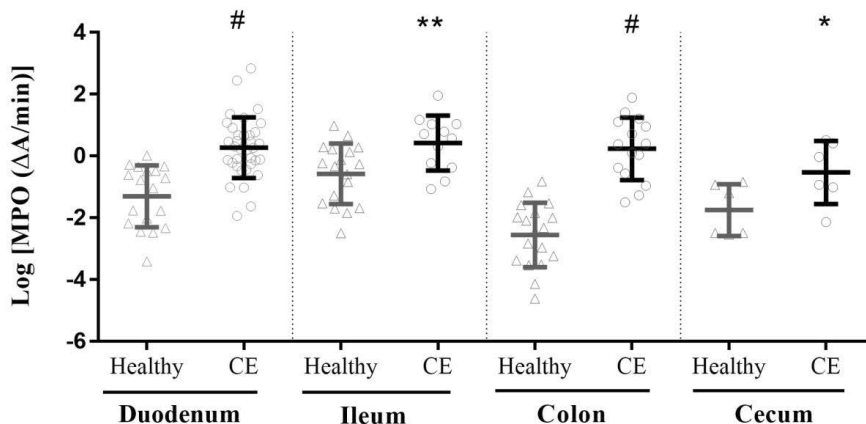


Fig. 15. Scatter plot displaying log-transformed intestinal mucosal MPO activity in CE dogs and healthy Beagles. Data are expressed as the mean \pm standard deviation. Individual data points are shown by triangles (samples from healthy Beagles) and circles (samples from CE dogs) CE: chronic enteropathies; * $P < 0.05$ vs. healthy cecum; ** $P < 0.01$ vs. healthy ileum; # $P < 0.0001$ vs. healthy duodenum and colon. (Study IV)

5.9 Histological examination

5.9.1 Healthy Beagle dogs (study I-II)

Based on the histological examination of the intestine, the median total WSAVA score of all samples was 0 (range 0–3) classifying all findings as insignificant. There were no histological abnormalities in the submucosa, the muscularis externa, and the serosa in any of the samples.

5.9.2 Healthy Beagle dogs (study III-IV)

Based on the histological examination of intestinal samples from healthy Beagles, the median total WSAVA score of all samples was 0 (range 0–4), classifying all findings as insignificant.

5.9.3 Mucosal MMP-2 and -9 activities in relation to histopathologic changes in dogs with CE (study III)

In the ileum, pro-MMP-2 activities were significantly higher in mucosal samples with normal epithelium ($P = 0.0317$) and lamina propria neutrophil score ($P = 0.0335$). There was a positive significant association between the active MMP-2 activities with the severity of lamina propria eosinophil scores in cecal samples ($P = 0.0339$). The association of active MMP-9 activities with the severity of lamina propria neutrophil scores in the duodenum ($P = 0.0398$), and the severity of lamina propria eosinophil infiltration scores in the cecum ($P = 0.0369$) were significantly positive. Active MMP-9 activities were also significantly higher in cecal samples with normal lamina propria lymphocytes/plasmacell scores ($P = 0.0491$).

5.9.4 Mucosal S100A12 concentrations in relation to histopathologic changes in dogs with CE (study IV)

Mucosal S100A12 concentrations showed a significant association with the severity of macrophage infiltration in the duodenum of dogs with CE (Table 5, $P = 0.0439$). In addition, the median (IQR) of duodenal mucosal S100A12 concentrations were significantly higher if the inflammatory infiltrate contained neutrophils 61.1 [19.51-179.60] vs. 42.72 [7.41-102.46] $\mu\text{g/L}$; $P = 0.037$). However, when S100A12 concentrations were compared among the severity groups (normal, mild, moderate, and severe) of neutrophilic infiltration in the duodenum, there was no significant association in dogs with CE (Table 5, $P = 0.1542$). However, mucosal S100A12 concentrations were numerically higher in those with mild, moderate, or severe neutrophilic infiltration than in those with no neutrophilic infiltration. In addition, no significant association was found between S100A12 concentrations and severity of neutrophilic infiltration in the ileum, colon, and cecum of dogs with CE. In the colon, mucosal S100A12 concentrations showed a significant association with the severity of epithelial injury and total histopathologic injury (Table 6, $P < 0.05$).

5.9.5 Mucosal MPO activity in relation to histopathologic changes in dogs with CE (study IV)

Higher mucosal MPO activity showed a significant association with severity of total histopathologic injury, epithelial injury, and eosinophilic infiltration in the

duodenum (Table 5, $P < 0.05$). In the duodenum of dogs with CE, mucosal MPO activity was numerically higher in samples with moderate and severe neutrophilic infiltration than in samples with mild or no neutrophilic infiltration (Table 5). However, these differences did not reach statistical significance ($P = 0.7326$).

Table 5. Association between mucosal S100A12 concentrations and MPO activities with severity of histopathological findings in the duodenum and ileum of dogs with CE. Statistical differences were calculated based on the log-transformed values of S100A12 and MPO. Untransformed values are reported as median (IQR).

Histopathological findings	N	Severity groups	S100A12 (µg/L) Median (IQR)	P value [#]	MPO (AA/min) Median (IQR)	P value [#]
Duodenum	35					
Total histopathological severity	35	Insignificant (n = 16) Mild (n = 15) Moderate (n = 4)	33.65 (7.41-155.9) 53.12 (10.07-164.85) 52.22 (42-179.6)	0.2240	0.9 (0.2-2.95) 1.49 (0.14-4.55) 7.54 (0.94-17.16)	<u>0.006*</u>
Morphological criteria	35					
Villus stunting	35	Normal (n = 20) Mild (n = 10) Moderate (n = 3) Severe (n = 2)	44.18 (7.41-155.9) 45.02 (19.51-179.6) 56.27 (42.72-164.85) 26.04 (10.07-42)	0.4090	1.23 (0.14-4.55) 1.21 (0.37-3.88) 2.16 (1.63-11.62) 8.85 (0.54-17.16)	0.1439
Epithelial injury	35	Normal (n = 30) Mild (n = 3) Moderate (n = 2)	48.68 (7.41-164.85) 28.94 (19.51-179.6) 34.38 (26.76-42)	0.9122	1.23 (0.14-11.62) 1.58 (0.94-2.9) 9.82 (2.48-17.16)	<u>0.0455*</u>
Crypt distension	35	Normal (n = 29) Mild (n = 5) Moderate (n = 1)	42.96 (7.41-164.85) 67.87 (19.75-179.6) 42.72	0.4156	1.29 (0.14-17.16) 0.94 (0.63-1.63) 11.62	0.0694
Lacteal dilation	35	Normal (n = 19) Mild (n = 10) Moderate (n = 6)	28.94 (7.41-155.9) 54.7 (12.49-144.29) 53.07 (42.72-179.6)	0.0889	1.24 (0.2-17.16) 1.07 (0.14-2.48) 2.81 (0.94-11.62)	0.0708
Mucosal fibrosis	35	Normal (n = 31) Mild (n = 4)	42.96 (7.41-179.6) 63.88 (10.07-93.02)	0.9191	1.36 (0.14-17.16) 0.83 (0.54-3.88)	0.6993
Inflammatory criteria	35					
Intraepithelial lymphocytes	35	Normal (n = 23) Mild (n = 10) Moderate (n = 2)	42.96 (7.41-164.85) 36.44 (11.53-179.6) 53.07 (44.42-61.71)	0.9381	1.24 (0.14-17.16) 1.12 (0.37-4.55) 2.41 (1.36-3.46)	0.7695
Lamina propria LPC	35	Normal (n = 2) Mild (n = 15) Moderate (n = 15) Severe (n = 3)	99.19 (42.48-155.9) 24.83 (7.41-164.85) 50.94 (10.07-179.6) 61.71 (42-93.02)	0.1963	0.53 (0.2-0.87) 1.24 (0.36-2.95) 1.36 (0.14-11.62) 3.46 (0.77-17.16)	0.0975
Lamina propria eosinophils	35	Normal (n = 22) Mild (n = 5) Moderate (n = 8)	58.99 (10.07-179.6) 19.75 (7.41-42.96) 43.33 (26.76-78.03)	0.0516	0.92 (0.14-3.46) 0.91 (0.64-2.95) 3.39 (1.22-17.16)	<u>0.0004*</u>
Lamina propria neutrophils	35	Normal (n = 20) Mild (n = 7) Moderate (n = 6) Severe (n = 2)	42.72 (7.41-102.46) 61.1 (19.51-155.9) 47.92 (24.83-179.6) 113.28 (61.71-164.85)	0.1542	1.07 (0.36-17.16) 1.49 (0.2-3.88) 1.4 (0.14-11.62) 2.81 (2.16-3.46)	0.7326
Lamina propria macrophage	35	Normal (n = 32) Mild (n = 3)	42.84 (7.41-164.85) 155.9 (44.2-179.6)	<u>0.0439*</u>	1.39 (0.14-17.16) 0.94 (0.2-1.36)	0.1786
Ileum	12					
Total histopathological severity	12	Insignificant (n = 8) Mild (n = 4)	106.93 (9.59-317.21) 125.92 (16.36-151.79)	0.9816	1.91 (0.34-2.83) 2.3 (0.79-7.01)	0.3260
Morphological criteria	12					
Villus stunting	12	Normal (n = 7) Mild (n = 5)	120.84 (9.59-317.21) 116.24 (16.36-151.79)	0.7443	2.04 (0.34-2.83) 1.77 (0.69-7.01)	0.3447
Epithelial injury	12	Normal (n = 10) Moderate (n = 2)	118.54 (9.59-317.21) 84.07 (16.36-151.79)	0.4956	1.91 (0.34-3.23) 4.18 (1.36-7.01)	0.2387
Crypt distension	12	Normal (n = 11) Moderate (n = 1)	116.24 (9.59-317.21) 120.84	0.6913	2.04 (0.34-7.01) 0.79	0.4692
Lacteal dilation	12	Normal (n = 8) Mild (n = 4)	106.93 (9.59-317.21) 125.92 (16.36-162.43)	0.9881	1.56 (0.34-2.83) 2.63 (0.79-7.01)	0.2034
Mucosal fibrosis	12	Normal (n = 12)	118.54 (9.59-317.21)	-	1.91 (0.34-7.01)	-
Inflammatory criteria	12					
Intraepithelial lymphocytes	12	Normal (n = 6) Mild (n = 6)	109.23 (9.59-162.43) 123.62 (16.36-317.21)	0.6809	1.42 (0.34-2.81) 2.3 (0.69-7.01)	0.1787

Lamina propria LPC	12	Normal (<i>n</i> = 4)	118.78 (47.08-162.43)	0.5561	2.12 (0.44-2.81)	0.9999
		Mild (<i>n</i> = 4)	68.6 (9.59-317.21)		1.81 (0.34-7.01)	
		Moderate (<i>n</i> = 4)	123.62 (47.32-151.79)		1.56 (0.69-3.23)	
Lamina propria eosinophils	12	Normal (<i>n</i> = 9)	130.99 (9.59-317.21)	0.2514	1.77 (0.34-3.23)	0.2019
		Mild (<i>n</i> = 2)	72.35 (47.08-97.62)		1.63 (0.44-2.81)	
		Moderate (<i>n</i> = 1)	16.36		7.01	
Lamina propria neutrophils	12	Normal (<i>n</i> = 9)	116.24 (9.59-317.21)	0.7430	2.04 (0.34-3.23)	0.5989
		Mild (<i>n</i> = 3)	120.84 (16.36-151.79)		1.36 (0.79-7.01)	
Lamina propria macrophage	12	Normal (<i>n</i> = 12)	118.54 (9.59-317.21)	-	1.91 (0.34-7.01)	-

n: sample size; IQR: interquartile range; LPC: lymphocytes/plasma cells; [#] ANOVA test after logarithmic transformation of original values; * statistically significant association

Table 6. Association between mucosal S100A12 concentrations and MPO activities with severity of histopathological findings in the colon and cecum of dogs with CE. Statistical differences were calculated based on the log-transformed values of S100A12 and MPO. Untransformed values are reported as median (IQR).

Histopathological findings	N	Severity groups	S100A12 (µg/L) Median (IQR)	P value [#]	MPO (ΔA/min) Median (IQR)	P value [#]
Colon	15					
Total histopathological injury	15	Insignificant (<i>n</i> = 7) Mild (<i>n</i> = 7) Moderate (<i>n</i> = 1)	49.01 (10.8-184.68) 111.4 (32.81-538.8) 1296.65	0.0274*	1.04 (0.22-3.37) 1.49 (0.38-4.08) 6.61	0.1280
Morphological criteria	15					
Epithelial injury	15	Normal (<i>n</i> = 8) Mild (<i>n</i> = 3) Moderate (<i>n</i> = 3) Severe (<i>n</i> = 1)	44.18 (10.8-184.68) 254.57 (89.16-536.8) 111.4 (32.81-211.53) 1296.65	0.0083*	1.28 (0.22-4.08) 1.49 (0.57-2.59) 0.68 (0.38-3.01) 6.61	0.4232
Crypt hyperplasia	15	Normal (<i>n</i> = 11) Mild (<i>n</i> = 3) Moderate (<i>n</i> = 1)	89.61 (10.8-536.8) 33.53 (32.81-1296.65) 44.9	0.8412	1.1 (0.22-3.37) 1.46 (0.68-6.61) 4.08	0.3437
Crypt dilation and distortion	15	Normal (<i>n</i> = 12) Mild (<i>n</i> = 3)	76.01 (10.8-536.8) 49.01 (32.81-1296.65)	0.5706	1.48 (0.22-4.08) 1.1 (0.68-6.61)	0.5858
Mucosal fibrosis and atrophy	15	Normal (<i>n</i> = 8) Mild (<i>n</i> = 5) Moderate (<i>n</i> = 1) Severe (<i>n</i> = 1)	53.97 (10.8-1296.65) 111.4 (43.45-254.57) 536.8 33.53	0.4267	0.86 (0.22-6.61) 2.59 (0.38-3.37) 1.49 1.46	0.9124
Inflammatory criteria	15					
Lamina propria LPC	15	Normal (<i>n</i> = 2) Mild (<i>n</i> = 9) Moderate (<i>n</i> = 3) Severe (<i>n</i> = 1)	312.98 (89.16-536.8) 49.01 (22.89-211.53) 44.9 (10.8-254.57) 1296.65	0.0554	1.03 (0.57-1.49) 1.1 (0.22-3.37) 2.59 (0.28-4.08) 6.61	0.4088
Lamina propria eosinophils	15	Normal (<i>n</i> = 9) Mild (<i>n</i> = 3) Moderate (<i>n</i> = 3)	63.04 (10.8-254.57) 33.53 (22.89-89.16) 536.8 (44.9-1296.65)	0.1076	1.04 (0.22-3.37) 1.46 (0.57-2.04) 4.08 (1.49-6.61)	0.1516
Lamina propria neutrophils	15	Normal (<i>n</i> = 10) Mild (<i>n</i> = 4) Moderate (<i>n</i> = 1)	46.96 (10.8-1296.65) 161.47 (33.53-254.57) 536.8	0.2448	1.07 (0.22-6.61) 2.02 (0.38-3.01) 1.49	0.9400
Lamina propria macrophage	15	Normal (<i>n</i> = 15)	63.04 (10.8-1296.65)	-	1.46 (0.22-6.61)	-
Cecum	6					
Total histopathological injury	6	Insignificant (<i>n</i> = 6)	160.38 (8.62-405.48)	-	0.68 (0.12-1.66)	-
Morphological criteria	6					
Epithelial injury	6	Normal (<i>n</i> = 3) Mild (<i>n</i> = 3)	34.02 (8.62-249.74) 351.79 (71.02-405.48)	0.2187	0.37 (0.12-1.66) 0.97 (0.4-1.51)	0.4642
Crypt hyperplasia	6	Normal (<i>n</i> = 5) Mild (<i>n</i> = 1)	71.02 (8.62-351.79) 405.48	0.3225	0.97 (0.12-1.66) 0.4	0.7163
Crypt dilation and distortion	6	Normal (<i>n</i> = 5) Mild (<i>n</i> = 1)	249.74 (8.62-405.48) 34.02	0.5229	0.98 (0.37-1.66) 0.12	0.0715
Mucosal fibrosis and atrophy	6	Normal (<i>n</i> = 6)	160.38 (8.62-405.48)	-	0.68 (0.12-1.66)	-
Inflammatory criteria	6					
Lamina propria LPC	6	Normal (<i>n</i> = 2) Mild (<i>n</i> = 4)	211.41 (71.02-351.79) 141.88 (8.62-405.48)	0.6222	1.24 (0.97-1.51) 0.38 (0.12-1.66)	0.2594
Lamina propria eosinophils	6	Normal (<i>n</i> = 3) Mild (<i>n</i> = 3)	34.02 (8.62-249.74) 351.79 (71.02-405.48)	0.2181	0.37 (0.12-1.66) 0.97 (0.4-1.51)	0.4642
Lamina propria neutrophils	6	Normal (<i>n</i> = 5) Moderate (<i>n</i> = 1)	71.02 (8.62-405.48) 351.79	0.4075	0.68 (0.12-1.66) 1.51	0.3664
Lamina propria macrophage	6	Normal (<i>n</i> = 6)	160.38 (8.62-405.48)	-	0.68 (0.12-1.66)	-

n: sample size; IQR: interquartile range; LPC: lymphocytes/plasma cells; [#] ANOVA test after logarithmic transformation of original values; * statistically significant association

5.10 Mucosal MMP-2 and -9 activities, S100A12 concentrations, and MPO activities in relation to CIBDAI and clinical outcome (study III-IV)

Based on treatment response after diet change or antibiotic or corticosteroid therapy, the outcomes of 30 dogs with CE were classified as follows: 10 dogs with FRE, 4 dogs with ARE, 13 dogs with SRE, and 3 dogs with SNRE. The median (range) of CIBDAI scores before and after treatment and the type of clinical outcome are summarized in Table 7. For pro- and active MMP-2 and -9, only active MMP-9 activities in the ileal mucosa had a strong positive correlation with the CIBDAI score before treatment in dogs with CE ($r = 0.71$, $P = 0.023$). In dogs with CE, no significant association was found between the mucosal pro- and active MMP-2 and -9 activities and the clinical outcome in each intestinal segment (data not shown, $P > 0.05$). In addition, no significant association was evident between mucosal S100A12 concentrations or MPO activities with CIBDAI scores before treatment or in the clinical outcome for each intestinal segment in dogs with CE (data not shown, $P > 0.05$).

Steroid non-responsive dogs (SNRE, $n = 3$) either died or were euthanized due to severe clinical signs and unfavorable response to treatments. When comparing duodenal samples, the median (range) of the CIBDAI score before treatment was higher in SNRE dogs ($n = 3$) than in SRE dogs ($n = 12$) (7 [4–7] vs. 4.5 [0–7]). Hypoalbuminemia (<20 g/L) was present in 1/3 SNRE and 2/12 SRE dogs. The median (range) of total histopathological injury score of SNRE dogs was 7 (5–7) and SRE dogs was 6 (1–11). The median (range) of the pro- and active MMP-2 and -9 activities in the duodenal mucosa of SNRE dogs compared to SRE dogs were as follows: pro-MMP-2 (0.01 [0–0.02] AU vs. 0.01 [0–1.66] AU), active MMP-2 (0 [0–0.01] AU vs. 0 [0–0.01] AU), pro-MMP-9 (0.07 [0.02–0.16] AU vs. 0.09 [0–3.86] AU), and active MMP-9 (0 [0–0.21] AU vs. 0 [0–0] AU). The median (range) of mucosal S100A12 concentrations and MPO activities in SNRE dogs compared to SRE dogs were as follows in the duodenum: (53.12 [19.51–93.02] g/L vs. 42.97 [12.49–179.6] g/L and (1.22 [0.77–1.58] Δ A/min vs. 1.21 [0.14–17.6] Δ A/min, respectively. The number of SNRE dogs was too low for statistical analysis other than descriptive comparison.

Table 7. Clinical outcome related to CIBDAI score before and after treatment in CE dogs.

Clinical outcome	Number of dogs	CIBDAI before treatment Median (range)	CIBDAI after treatment Median (range)
Food-responsive enteropathy (FRE)	10	4 (2-9)	0 (0-1)
Antibiotic-responsive enteropathy (ARE)	4	5.5 (1-6)	0 (0-0)
Steroid-responsive enteropathy (SRE)	13	4 (0-7)	0 (0-1)
Steroid non-responsive enteropathy (SNRE)	3	7 (4-9)	9 (4-10)

5.11 Mucosal MMP-2 and -9 activities, S100A12 concentrations, and MPO activities in relation to hypoalbuminemia in dogs with CE (study III-IV)

Out of 40 dogs, 36 (90%) had normoalbuminemia with a median (range) serum albumin concentration of 32.7 g/L (24.9-39 g/L). However, four dogs (10%) had hypoalbuminemia with a median (range) serum albumin concentration of 12.2 g/L (11-13 g/L). Duodenal mucosal biopsies were taken from the four hypoalbuminemic dogs and correlation between the mucosal MMP-2 and -9 activities, S100A12 concentrations, and MPO activities in the duodenum and hypoalbuminemia were evaluated.

In study III, there was no significant correlation between pro-MMP-2 ($r = -0.165$, $P = 0.345$), active MMP-2 ($r = -0.15$, $P = 0.389$), pro-MMP-9 ($r = -0.146$, $P = 0.402$), or active MMP-9 ($r = -0.024$, $P = 0.893$) activities in the duodenal mucosa and hypoalbuminemia in dogs with CE. In hypoalbuminemic compared to normoalbuminemic dogs with CE, the number [percentage] of positive samples in the duodenal mucosa was as follows: pro-MMP-2 (3/4 [75%] vs. 29/31 [93.5%]); active MMP-2 (1/4 [25%] vs. 9/31 [29%]; $P < 0.01$); pro-MMP-9 (3/4 [75%] vs. 31/31 [100%]); and active MMP-9 (1/4 [25%] vs. 1/31 [3.2%]). The median (range) of the pro- and active MMP-2 and -9 activities in the duodenal mucosa of hypoalbuminemic dogs with CE compared to normoalbuminemic dogs with CE was as follows: pro-MMP-2 (0.01 [0–0.017] AU vs. 0.01 [0–1.66] AU); active MMP-2 (0 [0–0.01] AU vs. 0 [0–0.02] AU); pro-MMP-9 (0.14 [0–0.74] AU vs. 0.05 [0.01–3.86] AU); and active MMP-9 (0 [0–0.21] AU vs. 0 [0–0.1] AU).

In study IV, the median (IQR) of mucosal S100A12 concentrations and MPO activities in hypoalbuminemic dogs with CE compared to normoalbuminemic dogs

were as follows: 128.94 [69.54-175.91] g/L vs. 42.72 [23.62-61.1] g/L and 1.55 [0.81-3.14] $\Delta A/\text{min}$ vs. 1.58 [0.81-2.9] $\Delta A/\text{min}$, respectively. Since the number of hypoalbuminemic dogs was too low for meaningful statistical analysis, we reported the results descriptively.

5.12 Correlation between S100A12 concentrations and MPO activities (study IV)

A moderate positive correlation between mucosal S100A12 concentrations and MPO activities was seen in dogs with CE and healthy Beagles combined ($r = 0.392$; $P < 0.0001$); this correlation was also observed in healthy Beagles alone ($r = 0.327$; $P = 0.011$). However, there was no correlation between mucosal S100A12 concentrations and MPO activities in dogs with CE alone ($p = 0.273$) or in the different intestinal segments from dogs with CE (duodenum: $p = 0.0949$; ileum: $p = 0.245$; colon: $p = 0.265$; cecum: $p = 0.325$).

6 DISCUSSION

6.1 Identification of mucosal MMP-2 and -9 activities in the intestine of healthy Beagles using gelatin zymography (study I)

In study I, MMP-2 and -9 were detected in four different sections of the intestinal mucosa of healthy Beagle dogs with a gelatin zymography method. The method has been used previously to study MMP-2 and -9 in human intestinal samples (Kleiner and Stetler-Stevenson, 1994; Baugh et al., 1999; Gao et al., 2005), but no known similar studies have yet been made from canine intestinal mucosal. In this study (I), stored intestinal mucosal samples were used, that had been collected during necropsy from 12 healthy Beagle dogs after finishing another non-related study to avoid the unnecessary use of laboratory animals for experimental studies. The collected samples were then stored at -80°C for later research. This approach complies with the principles of replacement, reduction, and refinement of animal experiments (Flecknell, 2002). It seems unlikely that the previous studies could have interfered with our results since all dogs had the same living and nutritional conditions and were clinically healthy at the time of euthanasia. In addition, the histological examination of the intestinal mucosa was a part of our study to investigate the possibility of subclinical intestinal inflammation which might have influence on the interpretation of the study results.

Gelatin zymography is a highly sensitive technique and is widely used in the determination of MMP-2 and -9 in tissue samples. This technique appears to be a useful method for the evaluation of intestinal mucosa samples, because it requires only a small piece of intestinal tissue sample (5 mg mucosa) which is the weight of about 1-2 endoscopically taken biopsies. This technique can detect even small amounts of MMP-2 and -9 from tissue-homogenization supernatant (Snoek-van Beurden and Von den Hoff, 2005). The gelatin zymography used here was described by Rajamäki et al. (2002), selecting both 10 and 25 µg of total protein in the extracted samples. The amount of 10 µg protein is often used in human IBD research for determination of MMPs by gelatin zymography. However, the majority of samples in this study that contained 10 µg of total protein showed no activity. It is likely that the levels of MMP-2 and -9 activities in healthy Beagles are too low to be detected in 10 µg protein samples in comparison to 25 µg protein samples. Therefore, only the results for samples containing 25 µg of total protein are presented in this thesis for the characterization of MMP-2 and -9 in the intestinal mucosa of healthy Beagles. To assess whether higher protein amounts lead to MMP detection in negative samples, larger protein amounts of 100 µg were tested, but these also gave negative results.

In the canine samples, the zymography method produced distinct bands on the gel which corresponded to the standards with human pro-MMP-2 and -9. No substantial difference was detected between the migration of these enzymes from human standards and intestinal mucosa samples obtained from healthy Beagles, which is in accordance with MMP-2 and -9 enzymes being highly conserved among species (Coughlan et al., 1998).

In study I, 65% and 48% of all samples showed no activity for pro-MMP-2 and -9, respectively, and the colon showed the lowest activity. One possible reason for the high amount of undetectable activity might be that the level of gelatinolytic activity of both MMPs was less than the detection limit of the gelatin zymography assay. Similar results were reported for normal human colon mucosa samples. In the study by Baugh et al. (1999), pro-MMP-2 and -9 were not detected in 20% and 56% of their samples, respectively, using gelatin zymography when examining colon mucosa from nine healthy human subjects. In study I, no intestinal samples showed gelatinolytic activity corresponding to the control bands of active MMP-2 and MMP-9. Similarly, Baugh et al. (1999) detected no active forms of MMP-2 and -9 in healthy human colonic mucosa samples.

Thirty-five percent (17/48) of the samples were positive for pro-MMP-2 activity in study I. In healthy Beagles, pro-MMP-2 activity was mostly seen in the small intestine. This might be due to high levels of epithelial cell turnover in the small intestine (Bergman et al., 2002). MMP-2 is commonly expressed in normal intestinal tissues and is believed to participate in the maintenance of collagen homeostasis and intestinal tissue remodeling (Gao et al., 2005; Sengupta and MacDonald, 2007). Pro-MMP-9 activity was detected in 52% (25/48) of the samples. Under normal conditions, MMPs including MMP-9 are expressed at low levels, usually in the pro-form, and when activated, they play a role in normal tissue ECM turnover, including intestinal tissue (Medina and Radomski, 2006).

Gao et al. (2005) reported the mean (\pm SEM) activities of pro-MMP-2 and pro-MMP-9 in normal colonic mucosa of humans to be < 2 AU with 0.64 ± 0.1 and 1.18 ± 0.19 AU, respectively. The majority of samples of study I (86%) also displayed pro-MMP-2 and -9 activities < 2 AU; however, some histologically unremarkable samples showed comparably high pro-MMP-2 and -9 activities > 2 AU, for which there is no clear explanation.

The activity of MMP-2 and MMP-9 in different parts of the intestinal mucosa of healthy Beagles has been determined in study I, laying the groundwork for further studies in canine chronic enteropathies. Pro-MMP-2 and -9 activities were found in the intestinal mucosa of the small and large intestines in healthy Beagles, mostly in the ileum. Active forms of MMP-2 and -9 were not detected in the intestinal mucosa of healthy Beagles. These findings raised the question whether MMP-2 and -9 are also involved in the pathogenesis of canine CE. Therefore, we collected intestinal mucosal biopsy samples from dogs with CE in study III to investigate their activities in the mucosa.

6.2 Mucosal MMP-2 and -9 activities in the intestine of dogs with CE (study III)

This thesis outlines the first known report on mucosal MMP-2 and -9 activities in the intestine of dogs with CE (Study III). In this study, the number of samples positive for pro- and active MMP-2 and pro-MMP-9 was higher in the mucosa of dogs with CE compared to healthy Beagles in all intestinal segments when determined by gelatin zymography. Similar findings were also reported for active MMP-2 and pro-MMP-9 in the colonic mucosa of humans with IBD compared to healthy controls (Baugh et al., 1999). In Study III, a significantly higher percentage of colonic mucosal samples in dogs with CE had pro-MMP-2 activity compared to healthy Beagles (86.7% vs. 16.7%). However, the percentage of samples with colonic mucosal pro-MMP-2 activity was the same in human patients with IBD and healthy controls, and activity was detected in 80% of the samples (Baugh et al., 1999). Active MMP-2 was detected in 46.7% of the colonic samples of dogs with CE, however, no activity was found in colonic mucosal samples of healthy Beagles. Similarly, Baugh et al. (1999) detect no active MMP-2 activities in the colonic mucosa of healthy humans, but did observe active MMP-2 activities in 35% of human IBD samples. Under normal conditions, MMP-2 is believed to participate in the maintenance of collagen homeostasis and intestinal tissue remodeling. In human IBD, MMP-2 has been reported to contribute to ECM remodeling and the degradation of basal membrane type IV collagen, leading to intestinal ulceration, epithelial damage, and/or fistula formation (Stallmach et al., 2000; Matsuno et al., 2003; McKaig et al., 2003; Gao et al., 2005; O'Sullivan et al., 2015). It appears that intestinal tissue turnover is increased during intestinal inflammation, tissue destruction, and healing processes, and demands greater MMP-2 activities in human IBD (Gao et al., 2005). In contrast, it has been reported that MMP-2 plays a protective role against tissue damage, possibly through the regulation of epithelial barrier function, in an MMP-2 knockout mouse model of IBD (Garg et al., 2006; Ravi et al., 2007; Garg et al., 2009). In our study (III), it appears that having higher activities of pro- and active MMP-2 in dogs with CE indicates the possible involvement of this enzyme in the pathogenesis of canine chronic enteropathies. In addition, we showed that the samples with higher pro-MMP-2 activities have an association with a normal epithelium and lamina propria without neutrophils in the ileum. Similar to the study on an MMP-2 knockout mouse model of IBD, it is possible that MMP-2 holds a protective role against tissue damage through the regulation of epithelial barrier function in dogs; however, more research is needed to clarify the role of MMP-2 in canine CE.

Mucosal pro-MMP-9 was detected in more than 90% of the samples in the intestinal segments of dogs with CE, while this form of enzyme was detected in 33% to 61% of the intestinal mucosal samples of healthy Beagles. Therefore, pro-MMP-9 activities are elevated in dogs with CE compared to healthy Beagles, which is similar

to the findings of Baugh et al. (1999) in humans with IBD. Nonetheless, no active form of MMP-9 was found in the mucosa of healthy Beagles in this study (study III), which is similar to the findings reported in the colonic mucosa of healthy humans (Baugh et al., 1999). In dogs with CE, the active form of MMP-9 was detected in 5.7%, 25%, 5.6%, and 50% of duodenal, ileal, colonic, and cecal samples, respectively. In the duodenal and cecal samples, a positive and significant association was evident between active MMP-9 activities and the severity of lamina propria neutrophilic and eosinophilic infiltration, with these cells being proposed as sources of MMP-9. In comparison to human IBD, the percentage of active MMP-9-positive samples in the colon of dogs with CE was much lower (55% vs. 5.6%) (Baugh et al., 1999). This difference could be due to the different pathophysiology of canine and human chronic colitis. A higher number of infiltrated neutrophils and more ulcerative lesions in humans with chronic colitis could be the possible reasons for the higher active MMP-9 activities in humans (Gao et al., 2005). In future studies, immunohistochemistry should also be included to assess the localization of MMP-2 and -9 in the intestinal mucosa and their correlation with intestinal pathologies in dogs.

A positive correlation was found between active MMP-9 activities in the ileal mucosa and CIBDAI score before treatment in dogs with CE, which is similar to the relationship between mucosal MMP-9 and disease activity index in a rat model of colitis (Oliveira et al., 2014), and between fecal MMP-9 and clinical activities of ulcerative colitis in humans (Farkas et al., 2015). However, due to the low number of active MMP-9 positive samples ($n = 3$) in the ileal samples of dogs with CE in our study, the results should not be over-interpreted. In the present study, we did not find a significant association between mucosal pro- and active MMP-2 and -9 activities and the type of clinical outcome in each intestinal segment in dogs with CE. One possible reason could be the low number of samples in some groups (Table 7) and because of assessing the relationship between the mucosal MMP -2 and -9 activities and type of clinical outcome in each intestinal segment of dogs with CE.

The association between an aberrant intestinal expression of MMP-2 and -9 and human IBD is now well established (O'Shea and Smith, 2014). MMP-2 and -9 have been investigated as biomarkers and diagnostic tool for human IBD. The level of fecal MMP-9 in UC patients correlates with disease activity and has recently been proposed as a biomarker of the disease. By measuring fecal MMP-9 levels, it was possible to distinguish UC from diarrhea predominant irritable bowel syndrome with 85% sensitivity and 100% specificity (Dabritz et al., 2014). Serum level of MMP-9 has also been shown as a potential tool in the prediction of CD activity status in children (Kofla-Dlubacz et al., 2012). In addition, MMP-2 and MMP-9 levels in urine of pediatric patients with IBD have been reported as useful novel non-invasive biomarkers to predict CD and UC independently in children (O'Shea and Smith, 2014). Our study is the first to demonstrate an upregulation of mucosal pro- and active MMP-2 and pro-MMP-9 in the intestine of dogs with CE. This is a promising indication that MMPs play a role in canine CE with the potential of being used as

biomarkers of active disease and disease severity. In the present study, however, we did not find a significant association between mucosal MMP-2 and -9 activities and the type of clinical outcome in dogs with CE which could be due to the low number of dogs with a certain clinical outcome (especially SNRE dogs, $n = 3$). In addition, the comparison between intestinal segments led to rather low case numbers when assessing the association between mucosal MMP-2 and -9 activities with the type of clinical outcome. Further studies with sufficient number of canine patients in each types of CE is needed to evaluate the relationship between MMP-2 and -9 activities and canine CE subtypes. MMP expression in the intestine or elsewhere in the body, e.g. serum or feces, may in the future help in properly differentiating disease subtypes and severity, and to enable tailored treatment choices for individuals.

A unique therapeutic option for human IBD is targeting MMPs which has been investigated in animal models of IBD. Inhibition of MMPs with non-selective inhibitors (e.g. Marimastat, Batimastat) has been shown to reduce mucosal damage and colitis induced by dextran sodium sulfate (Tyden et al.) in animal models (O'Shea and Smith, 2014). However, the development of MMP inhibitors in humans has been limited by their poor selectivity (O'Shea and Smith, 2014). To target MMPs, monoclonal antibodies against MMP-2 and MMP-9 have been developed and tested on murine models of IBD. These antibodies were significantly reducing the severity of the DSS induced colitis in mice (Sela-Passwell et al., 2011; O'Shea and Smith, 2014). However, due to the myriad roles of MMPs *in vivo* and their ubiquitous expression throughout the body, using monoclonal antibodies remains a serious concern and a barrier to their current therapeutic use in human IBD (O'Shea and Smith, 2014). Whether targeting MMPs using monoclonal antibodies in canine CE patients is beneficial for CE treatment is not known yet and needs to be studied.

It has been shown that treatment of human IBD with immunosuppressive medications could reduce the levels of MMPs in the intestinal mucosa and serum. Makitalo et al. (Makitalo et al., 2009) showed in people that the treatment of CD patients with anti-TNF- α therapy (infliximab or adalimumab) or with corticosteroids and other immunosuppressive drugs (methotrexate or azathioprine) decreased stromal MMP-9 and epithelial MMP-7 as assessed by immunohistochemistry methods. However, there are different reports regarding the response of MMP-9 to therapy in serum. Gao et al. (Gao et al., 2007) reported that serum levels of MMP-9 decrease in adults in response to infliximab therapy; however, Makitalo et al. (Makitalo et al., 2012) reported no significant changes in serum MMP-9 levels after therapy. In mice, magniferin, a bioactive compound of the mango, attenuated DSS induced colitis through directly reducing the activity of mucosal TNF- α and MMP-9 (Somani et al., 2016). Future studies in CE dogs should be planned as treatment follow up studies with repeated biopsies to evaluate the treatment effects of different medication especially immunosuppressive drugs such as prednisolone on the intestinal mucosal MMP-2 and -9 activities.

In study III, no significant correlations were evident between pro- and active MMP-2 and -9 activities in the duodenal mucosa and hypoalbuminemia in dogs with

CE. Since only 10% (4/40) of the dogs had hypoalbuminemia (<20 g/L), the number of samples was too low for an appropriate power of analysis and it could be the reason why we did not find significant correlations between MMP2 and 9 activities. In humans, to our knowledge, there has been so far no report studying MMP-2 and -9 activities in patients with protein losing enteropathies. In future prospective studies, the necessary number of cases of CE dogs with hypoalbuminemia should be estimated by using power analysis, for which the data of the current study are helpful.

6.3 Determination of mucosal S100A12 concentrations and MPO activities in the intestine of healthy Beagles (study II)

In study II, mucosal S100A12 concentrations and MPO activities in four different segments of intestine in healthy Beagle dogs were determined by an ELISA and a spectrophotometric method, respectively. The ELISA method was already validated for determination of S100A12 concentrations in the serum and fecal samples of healthy Beagles and dogs with IBD (Heilmann et al., 2016a), but not in canine intestinal mucosal extracts. Concentrations of S100A12 were higher in mucosal extracts from the ileum than in other segments of the intestine in healthy Beagles. S100A12 detection in the intestinal mucosa may reflect the number of neutrophils infiltrating the mucosa. Because of poor agreement between pathologists evaluating the number of duodenal neutrophils (Willard et al., 2010), no attempt was made to perform neutrophil count comparisons between the different intestinal sections. Therefore, the higher S100A12 concentrations in the ileum may be due to the high number of S100A12-expressing cells (neutrophils and macrophages) within the ileal mucosa. Heilmann et al. reported variations in the fecal concentrations of S100A12 in healthy dogs (Heilmann et al., 2011a). An irregular distribution of S100A12-expressing cells within the gastrointestinal mucosa or variation in the concentration of fecal proteins due to variations in the gastrointestinal passage were suggested as possible reasons for this variation (Heilmann et al., 2011a). Moreover, Heilmann et al. reported a similar variation in other fecal markers in healthy dogs (Heilmann et al., 2008; Heilmann et al., 2011b). In study II, the S100A12 concentration in all mucosal extracts ranged from 2.5–237.6 µg/L. Similar variations in the S100A12 concentration were reported for normal duodenal and cecal mucosal biopsies in humans (Leach et al., 2007). In a study by Leach et al., duodenal and cecal biopsies were cultured and supernatants were collected for the determination of S100A12 using an ELISA method. In that study, the S100A12 concentration ranged from 7–36 µg/L in the culture supernatants of duodenal and cecal mucosal biopsies obtained from 33 non-IBD control children (Leach et al., 2007). A similar variation was also found in the concentration of calprotectin, another member of the S100/calgranulin

protein family, ranging from 16–511 µg/L in the culture supernatants of duodenal and cecal mucosal biopsies (Leach et al., 2007).

In study II, a spectrophotometric method to measure MPO activity in the intestinal mucosa in healthy Beagles was successfully established and validated for the first time. Mucosal MPO activity was higher in the ileum, jejunum, and duodenum than in the colon of healthy Beagles. Moreover, its activity was higher in the jejunum than in the duodenum. The detection of MPO in the intestinal mucosa could reflect the number of granulocytes infiltrating the mucosa. Variations in the MPO activity in different parts of the intestine in healthy Beagles could be explained by the disparity in the distribution of MPO-releasing cells, such as the neutrophils and macrophages. Several previous studies have investigated the distribution of immune cells in the intestines of healthy dogs and cats (German et al., 1999; Waly et al., 2001; Kleinschmidt et al., 2008; Siddique et al., 2009; Marsilio et al., 2011). However, the results did not allow for conclusions concerning the distribution of MPO, since the distribution of neutrophils as the primary source of MPO was not studied. Macrophages, as a possible source of MPO, were detected using the antibody MAC387 in two studies of the intestines of normal cats (Waly et al., 2001) and dogs (Siddique et al., 2009). The authors concluded that the number of MAC387 positive cells was higher in the ileum than in other segments of the intestine. However, no known study has been performed to address the distribution of the MPO enzyme in different parts of the normal intestinal mucosa of dogs or any other species.

Several studies reported increased levels of both S100A12 and MPO in human patients with IBD (Foell et al., 2003b; de Jong et al., 2006; Kayo et al., 2006; Kaiser et al., 2007; Leach et al., 2007; Peterson et al., 2007; Foell et al., 2008; Sidler et al., 2008; Wagner et al., 2008; Hegazy and El-Bedewy, 2010; Dabritz et al., 2013). However, studies determining S100A12 and MPO in intestinal mucosal samples from dogs suffering from CE remain unreported. Fecal S100A12 concentrations have been reported to be increased in dogs with CE compared to healthy Beagles (Heilmann et al., 2014a). Whilst using non-invasive biomarkers is more appropriate in determining the presence of gut inflammation, measuring the fecal S100A12 concentration does not allow for the assessment of the specific part of the intestine from which the S100A12 sample originates. Fecal S100A12 may be valuable for screening dogs suspected of having chronic enteropathies and for monitoring disease activity, thus reducing the need for endoscopy during disease follow-up. Increases in fecal S100A12 are, therefore, viewed as indicative of endoscopy, rather than being used as an alternative to endoscopy for diagnosis (de Jong et al., 2006). In study II, laboratory methods were validated to determine the biomarkers in tissues from their proposed origin. Both mucosal S100A12 and MPO may be valuable markers complementing invasive diagnostic measures when used in combination with intestinal mucosal histology. This is an important basis for the study of the role of such markers in the pathogenesis of canine CE.

6.4 Mucosal S100A12 concentrations and MPO activities in the intestine of dogs with CE (study IV)

In study IV, we reported mucosal S100A12 concentrations and MPO activities in the intestine of dogs with CE. The results of study IV showed that the mucosal S100A12 concentrations are significantly higher in dogs with CE than in healthy Beagles in the duodenum and colon (Fig. 13). In the ileum and cecum, mucosal S100A12 concentrations were numerically higher in dogs with CE than those in healthy Beagles. However, this difference did not reach statistical significance (Fig. 13). The results of our investigation in the duodenum are consistent with those from Leach et al. in humans with IBD (Leach et al., 2007). In that study, duodenal and cecal biopsies of children with IBD (including UC, CD, or unclassified IBD) were cultured and supernatants were collected for ELISA determination of S100A12 concentrations. Similar to the results of study IV, children with UC, CD, or unclassified IBD had significantly higher S100A12 concentrations in the culture supernatants of duodenal and cecal mucosal biopsies than those in non-IBD control children (Leach et al., 2007). In another similar study in humans, Foell et al. (Foell et al., 2008) also found higher S100A12 concentrations in culture supernatants of colonic and ileal biopsy samples in patients with active CD than in those patients with inactive CD or healthy controls. In addition, higher S100A12 concentrations were found in culture supernatants of colonic samples in patients with active UC than in those patients with inactive UC or healthy controls. Similar to Foell et al. (2008), significantly higher S100A12 concentrations were evident in colonic samples from dogs with CE than those in healthy Beagles (Study IV). However, in contrast to humans with CD, despite higher mucosal S100A12 concentrations in ileal samples of dogs with CE compared with healthy Beagles, this difference did not reach statistical significance. Heilmann et al. (Heilmann et al., 2014a) previously reported significant upregulation of fecal S100A12 concentration in dogs with CE compared with healthy Beagles, which is consistent with our results in the duodenum and colon (study IV).

Colonic mucosal S100A12 concentrations significantly associated with the severity of epithelial injury and total histopathological injury in dogs with CE (Table 6). When S100A12 binds to RAGE, an inflammatory response occurs due to the production of pro-inflammatory cytokines via activation of NF- κ B, which ultimately leads to tissue damage (Hofmann et al., 1999; Foell et al., 2007; Pietzsch and Hoppmann, 2009). In the current study, higher mucosal S100A12 concentrations were found if the inflammatory infiltrate had a neutrophil or macrophage

component. This is consistent with the findings in human patients with IBD (Foell et al., 2003b). However, since only 3 dogs had mild macrophage infiltration, the results should not be over interpreted and rather be a sign that more research needs to be performed with macrophage specific staining. In dogs with CE, however, Heilmann et al. (Heilmann et al., 2014a) did not observe a significant association between fecal S100A12 and the presence of neutrophils and macrophages in the intestinal mucosal biopsies or with the site(s) of inflammatory lesions. It is challenging to correlate fecal S100A12 concentrations to the site(s) of inflammatory lesions and cellular infiltrates within the GI tract, as it is unclear from which part of the intestine the S100A12 protein originates. Fecal S100A12 can be considered as reflective for mucosal S100A12 concentration giving an indication for the need to localize the site of the inflammatory lesions within the GI tract and to identify the areas most affected by the disease process. In addition to standard histology, mucosal biomarkers could provide valuable information concerning localization, severity and possible pathogenesis of inflammatory processes.

In study IV, mucosal MPO activity was significantly higher in all studied segments of the intestine of dogs with CE than the corresponding activity in healthy Beagles (Fig. 15). Similar results were also observed in the colonic mucosa of humans with IBD (Kruidenier et al., 2003; Kayo et al., 2006; Hegazy and El-Bedewy, 2010; Hansberry et al., 2017) and in animal models of human IBD (Kim et al., 2012; Li et al., 2016; Lv et al., 2017). In addition to the bactericidal activity of MPO products in activated phagocytes, there is considerable evidence that inappropriate or excessive stimulation of oxidant formation by this enzyme can result in host tissue damage (Davies, 2011). In the present study, mucosal MPO activity showed a significant association with severity of epithelial injury and total histopathological injury in the duodenum of dogs with CE (Table 5), which is consistent with findings in humans with IBD (Kruidenier et al., 2003; Kayo et al., 2006) and also in animal models of human IBD (Kim et al., 2012). The most severe histopathological injuries observed in mucosal samples with higher MPO activity could be explained by MPO-induced oxidative tissue damage in the inflamed mucosa. In addition, mucosal MPO activity showed a significant association only with eosinophil infiltration but not with neutrophil infiltration in the duodenum. Although MPO activity levels were numerically higher in samples with moderate and severe neutrophilic infiltration than those with mild or no neutrophilic infiltration in the duodenum of dogs with CE, these differences were not statistically significant (Table 5). In future studies, immunohistochemistry can be used to localize S100A12 and MPO cellular origin more specifically in the intestinal mucosa of dogs with CE.

In study IV, no significant association was found between mucosal S100A12 concentrations or MPO activities in each intestinal segment with CIBDAI score before treatment or CE type (ie, FRE, ARE, SRE, or SNRE). Similarly in children with IBD, mucosal S100A12 concentrations were not associated with the pediatric Crohn's disease activity index (PCDAI) (Leach et al., 2007). However, these

findings contrast with the study of Heilmann et al. (Heilmann et al., 2014a), who found a significant correlation between fecal S100A12 and the canine chronic enteropathy clinical activity index (CCECAI). It should be taken into consideration that CIBDAI or CCECAI include a number of subjective elements and consequently do not always represent the actual inflammatory burden, which may lead to discordance between the results of this study with others. In contrast to the reported results (Study IV), another study by Heilmann et al. (Heilmann et al., 2016b) revealed elevated fecal S100A12 concentrations in dogs with SRD compared with those with FRD or ARD. Elevated fecal S100A12 concentrations were also observed in SNRD dogs compared with those in dogs that underwent complete remission. One of the limitations of study IV is that we did not measure S100A12 concentrations in the feces of dogs with CE to assess for any associations between fecal S100A12 concentrations and types of clinical outcome. Accordingly, direct comparisons between our results and those with of Heilmann et al. (Heilmann et al., 2016b) are not possible. One possible reason why a significant association between these biomarkers and clinical outcome was not observed might be the small number of samples from dogs with a certain clinical outcome (especially SNRE dogs, $n = 3$) (Table 3). Also the comparison between intestinal segments led to rather low case numbers when assessing the association between mucosal S100A12 concentrations and MPO activities with the type of clinical outcome.

Duodenal S100A12 concentrations were higher in four hypoalbuminemic dogs with CE. However, due to the low number of hypoalbuminemic dogs in our study, the clinical significance of our results is not clear. However, Heilmann et al. did not find a significant association between fecal S100A12 concentrations and hypoalbuminemia (<20 g/L) in dogs with CE despite numerically higher fecal S100A12 concentrations in dogs with hypoalbuminemia (Heilmann et al., 2014a). Our findings suggest that further studies are warranted in more severely affected patients and should include measurements of both fecal and mucosal S100A12 concentrations.

In study IV, a moderate positive correlation was observed between mucosal S100A12 concentrations and MPO activities in dogs with CE and healthy Beagles combined and also in healthy Beagles alone. However, no correlation was found in dogs with CE alone and also when analyzing different intestinal segments of dogs with CE. In pediatric studies, a moderate positive correlation was found between mucosal S100A12 and calprotectin concentrations only in non-IBD controls but not in patients with IBD (Leach et al., 2007). Given that both S100A12 and MPO are derived predominantly from neutrophils, a correlation between the two biomarkers in dogs with CE was expected. Possible reasons for not having this correlation could be that two different methods were used to measure S100A12 concentrations and MPO activities and different functional pathways of these biomarkers in intestinal inflammation.

In the study III and IV, we used stored intestinal mucosal samples from both diseased and healthy Beagle dogs. In the case of healthy Beagle dogs, the samples were collected during post-mortem examinations after finishing other non-related studies to avoid the unnecessary use of laboratory animals for experimental proposes. This methodology complies with the principles of replacement, reduction and refinement of animal experiments (Flecknell, 2002). As this approach requires long-term stability of the measured analytes, we checked the MPO activity in 14 randomly chosen mucosal supernatant samples before and after 3 years of storage at -80°C. The median (range) of MPO activity at the first measurement (0.35 Δ A/min (0.01-1.92)) and after 3 years of storage at -80°C (0.35 Δ A/min (0.004-2.19)) were not statistically different ($P = 0.101$), which shows long-term stability of MPO activity in mucosal supernatant samples. Concentrations of S100A12 in fecal extracts stored frozen at -80°C have also been shown to be stable for years (Heilmann RM, unpublished data) and it is reasonable to assume that the same holds true for mucosal specimens. In addition, in our study, the control dogs were intact and older Beagles, however, it is rather unlikely that breed, gender, age, or being castrated or intact has an influence on our results. Age had also no influence on colonic mucosal MPO activity in baboons (Tran and Greenwood-Van Meerveld, 2013).

6.5 Limitations

6.5.1 Study I and II

In some occasional histological samples in study I, it was impossible to interpret epithelial changes due to processing artifact. However, since several samples were used per location for histology, there was enough epithelial layer on the majority of samples from the same intestinal segment of every dog to allow a final interpretation. The submucosa, muscularis, and serosa were not affected by artifacts.

6.5.2 Study III and IV

One of the limitations in study III and IV was too low number of CE dogs with hypoalbuminemia (<20 g/L) for an appropriate power of analysis. In future prospective studies, the necessary case numbers of CE dogs with hypoalbuminemia should be prospectively estimated.

In addition, S100A12 concentrations were not measured in the feces of dogs with CE to assess for any associations between fecal S100A12 concentrations and types

of clinical outcome (study IV). Another limitation of our study is that the cobalamin and folate concentrations were not measured in a sufficient number of dogs with CE to allow statistical analysis. Therefore, future prospective studies need to include a variety of laboratory data to assess possible associations/correlations. Finally, the treatment follow up was not standardized in study III and IV which should be considered when planning future studies.

6.6 Further research

- Further research is needed to assess the localization of MMP-2 and -9 in canine intestinal mucosa, their presence and activity level in advanced CE with intestinal protein loss, and their relation to the other inflammatory markers in canine chronic enteropathies.
- In the future, it will also be necessary to focus more on advanced cases with intestinal protein loss, a patient group that was underrepresented in this study and also looking for other MMPs.
- MMP-2 and -9 activities in fecal samples should be determined to study their possible usefulness as non-invasive biomarkers of intestinal inflammation (Annahazi et al., 2013; Pujada et al., 2016).
- In humans, several studies have investigated the role of other MMPs (e.g., MMP-1, MMP-3, MMP-7, MMP-10) and their inhibitors (e.g., TIMP-1, TIMP-3) in the pathogenesis of human IBD (von Lampe et al., 2000; Matsuno et al., 2003; Ravi et al., 2007; Mäkitalo, 2010). Studies are therefore warranted to determine those MMPs and TIMPs activities also in the intestinal mucosa of canine CE.
- In the future, it is needed to assess the value of measuring mucosal S100A12 concentrations and MPO activities in clinical practice, and also their relation to fecal S100A12 and MPO as well as other inflammatory markers in dogs with chronic enteropathies.

7 CONCLUSIONS

Based on these studies, the following conclusions can be drawn:

1. Based on the results of Study I and reports from studies in human patients with IBD, zymography presents as an appropriate method to evaluate MMP-2- and -9 profiles in canine intestinal mucosa.
2. Study II showed that S100A12 and MPO are reliably detectable in canine intestinal mucosa using the ELISA and spectrophotometric methods. The assays used appeared to be sufficient to further evaluate the role of S100A12 and MPO in the pathogenesis of canine chronic enteropathies.
3. Study III showed an upregulation of mucosal pro- and active MMP-2 and pro-MMP-9 in the intestine of dogs with CE compared to healthy Beagles. Compared to human patients with IBD, the active form of MMP-9 has been detected in a rather small number of canine patients which could be due to low number of granulocytes found in the intestinal mucosa of dogs with CE. The results, however, provide supporting evidence for the possible involvement of MMP-2 and -9 in the pathogenesis of canine CE.
4. The results of Study IV showed that both mucosal S100A12 concentrations and MPO activities are significantly increased in the duodenum and colon of dogs with CE, with mucosal MPO being also increased in the ileum and cecum. In study IV, mucosal S100A12 concentrations in dogs with CE correlated with the severity of epithelial injury and total histopathological injury in the colon; and with the presence of neutrophils and macrophages in the duodenal mucosa or with hypoalbuminemia. In addition, mucosal MPO activity is associated with the severity of epithelial injury and total histopathological injury in the duodenum of dogs with CE. The results also provide supporting evidence for the utility of mucosal S100A12 and MPO as potential diagnostic biomarkers in dogs with CE.

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